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Midkine, a heparin-binding cytokine, plays key roles in intraperitoneal adhesions

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Abstract

Midkine is a heparin-binding cytokine or growth factor and promotes the migration of inflammatory leukocytes. Upon partial hepatectomy, adhesion of the intestine was less severe in midkine-deficient mice than in wild-type mice. In a newly developed assay, in which the omentum adhered to the injured peritoneal wall, the incidence of adhesion in the deficient mice was reduced to 20% of that in the wild-type mice. Administration of midkine to the deficient mice increased the frequency of adhesion. The area of adhesion was also reduced to 8.3% in the deficient mice. The extent of migration of macrophages and neutrophils in the omentum around the adhesive region was reduced in the deficient mice. Therefore, midkine was concluded to play important roles in the formation of intraperitoneal adhesions, at least partly by promoting the migration of macrophages and neutrophils to the omentum.

Keywords: Abdominal operation; Cell migration; Cytokine; Intraperitoneal adhesions; Inflammatory cells; Knockout mice; Macrophages; Midkine; Neutrophils

Intraperitoneal adhesions between organs or organs and peritoneal walls occur in more than 90% of cases involving major abdominal operations [1]. These adhesions cause a narrowing of digestive tracts, ileus, and female infertility, and constitute one of the major causes of postoperative morbidity. Inflammations caused by mechanical stimuli are the principal causes of intraperitoneal adhesions. Recently, membranes, which are left in the peritoneal cavity and become absorbed, have been introduced as effective means to prevent intraperitoneal adhesions [2]. However, only adhesions of organs separated by membranes can be prevented and other effective means to prevent intraperitoneal adhesions are urgently needed. Various experiments have been performed to prevent adhesions [3–14]. New candidates for preventive therapeutics include polysaccharides and an inhibitor of collagen synthesis.

In spite of the practical importance of preventing adhesions, the study on the mechanism of adhesions has progressed relatively slowly. Inflammatory leukocytes should play important roles, but very few studies have been performed on the relationship between cytokines and intraperitoneal adhesions [15–18]. The administration of anti-macrophage chemotactic protein-1 (MCP-1) lowered the degree of intraperitoneal adhesions by 50% in a mouse model [17]. In the present study, we introduced a new model of postoperative adhesions and also employed knockout mice deficient in a cytokine gene. Consequently, we obtained evidence that midkine (MK), a heparin-binding cytokine or growth factor [19,20], is fundamentally involved in the formation of intraperitoneal adhesions.

MK is a basic, cysteine-rich polypeptide of 13 kDa having about 50% sequence identity with pleiotrophin

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(PTN)/HB-GAM [19,21–23]. MK promotes the growth, survival, differentiation, and migration of various target cells [19]. Most importantly, MK promotes the migration of neutrophils and macrophages directly and also indirectly through the induction of chemokines [24–26]. In mice deficient in the MK gene (*Mdk*), neointima formation [24] and nephritis [26] upon ischemic injury were significantly suppressed as compared to wild-type mice. Identification of a key factor involved in intraperitoneal adhesions is important to design a new approach to prevent the adhesion and also to understand the genetic difference in the severity of postoperative adhesions.

Materials and methods

Animal experiments. Mdk (−/−) mice were produced as described previously [27]. The heterozygotes were backcrossed nine times to C_{57}BL/6J mice. Then, they were mated to each other to yield *Mdk* (−/−) with the C_{57}BL/6J background. C_{57}BL/6J mice were used as wild-type controls. Female mice aged 4 months were used. They were anesthetized by intraperitoneal injection of sodium pentobarbital (Abbott Laboratories, North Chicago, IL) (40 mg/kg) and the abdomen was opened. On the inside of the left upper abdomen, a 5 × 5 mm abrasion was made with a No. 11 surgical knife. After the bleeding had been stopped by bipolar cautery, the abdomen was closed with 5-0 Nylon suture. After 7 days, the abdomen was re-opened under sodium pentobarbital anesthetization as described above, and the area of adhesion of the omentum to the injured site was determined using Scion Image image analysis software (Scion, City, MA). In the pump study, human MK in saline (1 mg/ml) or bovine serum albumin (Wako Pure Chemical Industries, Osaka, Japan) in saline (1 mg/ml) was infused using an osmotic pump (Alza, Palo Alto, CA) into *Mdk* (−/−). The pumps, which were implanted under the skin on the back, infused a total of 90 μl continuously over a period of 7 days. Human MK was produced in yeast and was kindly provided by Dr. S. Sakuma, Cell Signals. For partial hepatectomy, mice were anesthetized with sodium pentobarbital at a dose of 40 mg/kg body weight, and the right and left medial lobes, and the left lateral lobe, were surgically removed. Adhesions were examined after 2 weeks. All experiments were approved by the Animal Ethics Committee of Nagoya University.

Immunochemical analysis. Anti-mouse or human MK was produced as described [28]. The site of MK expression was revealed by immunohistochemical analysis [28] using affinity-purified rabbit anti-mouse MK or anti-human MK as the primary antibody and horseradish peroxidase-labeled affinity-purified goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grobe, PA) as the secondary antibody. The staining was visualized with diaminobenzidine tetrahydrochloride (Amersham Pharmacia Biotech, Tokyo, Japan). Fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Sigma, St. Louis, MO) was also used as secondary antibody. Staining for the macrophage marker or neutrophil marker was performed in the same way: the sections were stained with a monoclonal antibody to rat anti-mouse monocyte-macrophage marker F4/80 (Serotec, Oxford, UK) or to rat anti-mouse neutrophil marker 7/4 (Serotec), and then incubated with horseradish peroxidase-labeled goat anti-rat IgG (Jackson ImmunoResearch Laboratories). Human macrophages were detected by using CD68 Ab-3 (NeoMarkers, Fremont, CA) as the first antibody. The number of cells in a field under 400× magnification was counted. A total of 12 fields were examined and the average value was obtained. Four mice of each genotype were examined at each time point and the average value is shown with SD. Mouse peritoneal macrophages were isolated as described previously [29].

Human omentums were obtained intraoperatively from patients who underwent autoplasty of the omentum to various underlying diseases. The specimens were taken from the excess part. Patients with underlying bowel disease (i.e., inflammatory bowel disease) or who had received the operation of abdomen previously were excluded. Informed consent was obtained from all patients for participation in this study in accordance with the standards at Nagoya University.

Western blotting. The expression of MK was determined by Western blot analysis of heparin-binding proteins, which were derived from 1.7 mg of extract, as reported [28].

Results

Decreased intraperitoneal adhesions in Mdk (−/−) mice

Upon performing a partial hepatectomy, we found that adhesion of the intestine was less severe in *Mdk*

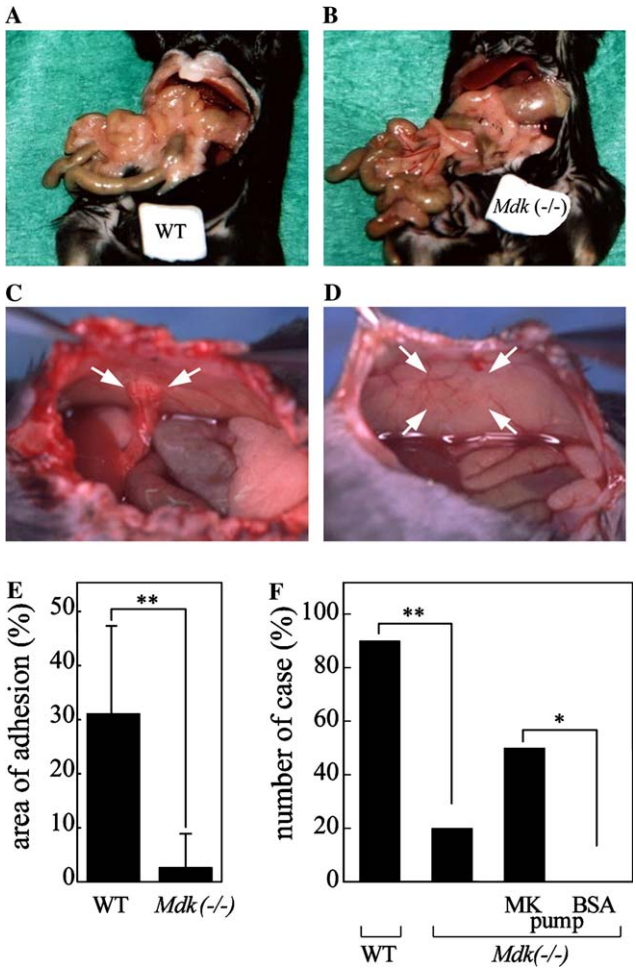

Fig. 1. Decreased intraperitoneal adhesion in *Mdk* (−/−) mice. (A,B) Intestinal adhesion observed 2 weeks after partial hepatectomy. Representative cases observed in WT ($n = 10$) (A) and *Mdk* (−/−) ($n = 10$) (B) mice are shown. (C,D) Adhesion of the omentum to the injured peritoneal wall. The area indicated by the arrows was abraded by operation and the adhesion was examined after 7 days. Representative cases for WT (C) and *Mdk* (−/−) mice (D) are shown. (E,F) Quantitative estimation of the decreased adhesion of the omentum to the injured peritoneal wall in *Mdk* (−/−) mice. (E) Area of adhesion in WT ($n = 10$) and *Mdk* (−/−) ($n = 10$) mice shown as a percentage of the defected area. (F) Incidence of adhesion in WT and in *Mdk* (−/−) mice, which were left untreated ($n = 10$) or infused with MK ($n = 10$) or bovine serum albumin (BSA) ($n = 10$). $^*p < 0.05$; $^{**}p < 0.01$. Statistical significance was evaluated with Student's *t* test in (E) and Fisher's exact test in (F).

(−/−) than wild-type (WT) mice. Adhesion occurred in all WT mice (Fig. 1A), but only about 50% of *Mdk* (−/−) mice (Fig. 1B). To perform a detailed analysis, we developed an omentum adhesion assay. A wound was produced in the peritoneal wall of *Mdk* (−/−) or WT mice. In WT mice (Fig. 1C) 7 days after the injury, the omentum adhered to the peritoneal wall, while in *Mdk* (−/−) mice (Fig. 1D) no or very little adhesion occurred. The average area of adhered omentum (Fig. 1E) and the incidence of adhesion (Fig. 1F) were significantly lower in *Mdk* (−/−) than WT mice. When MK was subcutaneously supplied to *Mdk* (−/−) mice by an osmotic pump, the adhesion was significantly restored; the supply of bovine serum albumin had no effect (Fig. 1F). This finding confirmed that the loss of MK indeed contributed to the decreased adhesion in *Mdk* (−/−) mice. From all these results we conclude that MK is fundamentally involved in intraperitoneal adhesions.

Localization of MK in the omentum

During the course of the adhesion, MK was predominantly expressed in the omentum; an example of the immunohistochemical staining is shown in Fig. 2A. MK was mainly located in macrophage-like cells and blood vessels. That MK is expressed in macrophages was confirmed not only by parallel staining with anti-MK antibody and anti-macrophage markers (Fig. 2B), but also by examining MK expression in isolated

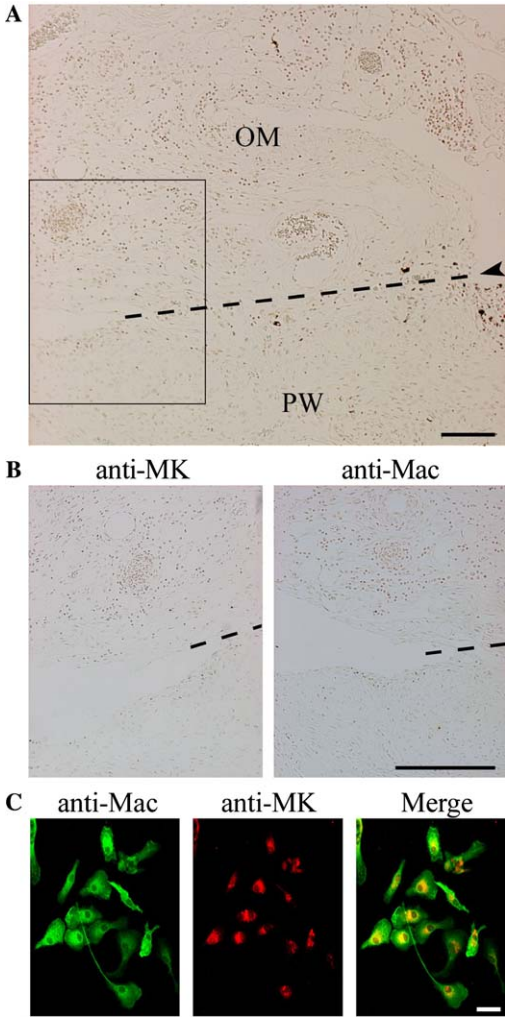

Fig. 2. Localization of MK in the mouse omentum. (A) Anti-MK staining of the adhesive region on Day 5. The dashed line shows the site of adhesion. OM, omentum; PW, peritoneal wall. Bar, 100 μm. (B) The boxed area in (A) was enlarged. Anti-MK, staining with anti-MK; anti-Mac, staining with anti-monocyte-macrophage marker. Bar, 100 μm. (C) Staining of peritoneal macrophages with anti-macrophage marker or anti-MK antibody. Bar, 50 μm.

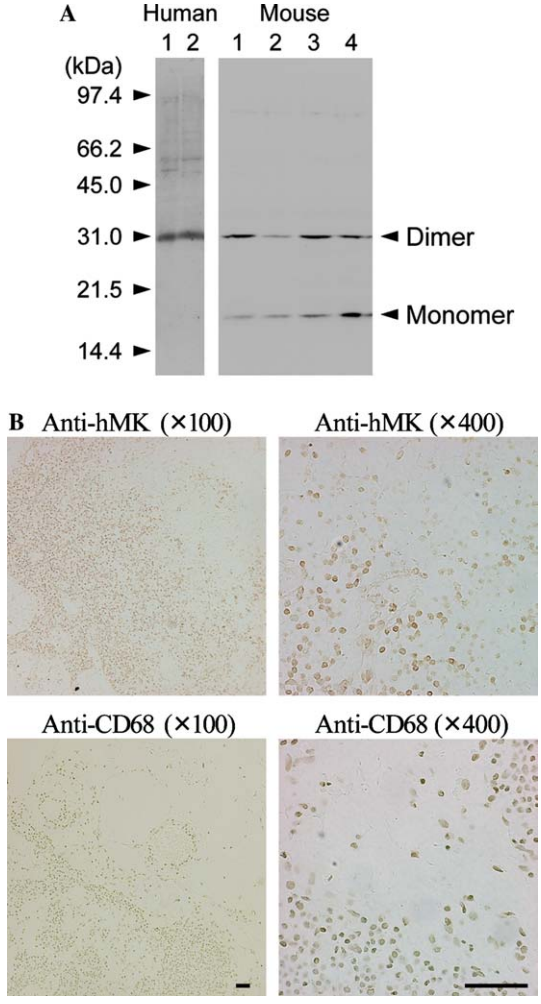

Fig. 3. Identification of MK in the mouse and human omentum. (A) Western blot analysis to identify MK in the omentum. Human, human omentum; Mouse, mouse omentum. Lanes 1 and 2 of "Human" show samples from two patients. Lanes 1, 2, 3, and 4 of "Mouse" show samples taken from Days 0, 3, 5, and 7 after injury. Although MK monomer has molecular weight of 13 kDa, it migrates as a 17 kDa molecule due to strong basic charge. (B) Immunohistochemical analysis of the human omentum. Anti-hMK, staining with anti-human MK; anti-CD68, staining with anti-CD68. Left (above, below), 100× magnification; right (above, below), 400× magnification. Bar, 50 μm.

peritoneal macrophages (Fig. 2C). Western blot analysis revealed that MK from the omentum consisted of both the monomer and the dimer (Fig. 3A). The human omentum contained similar levels of MK, although only the dimer was found (Fig. 3A). In the human omentum, MK positive cells were also macrophages positive for CD68 (Fig. 3B).

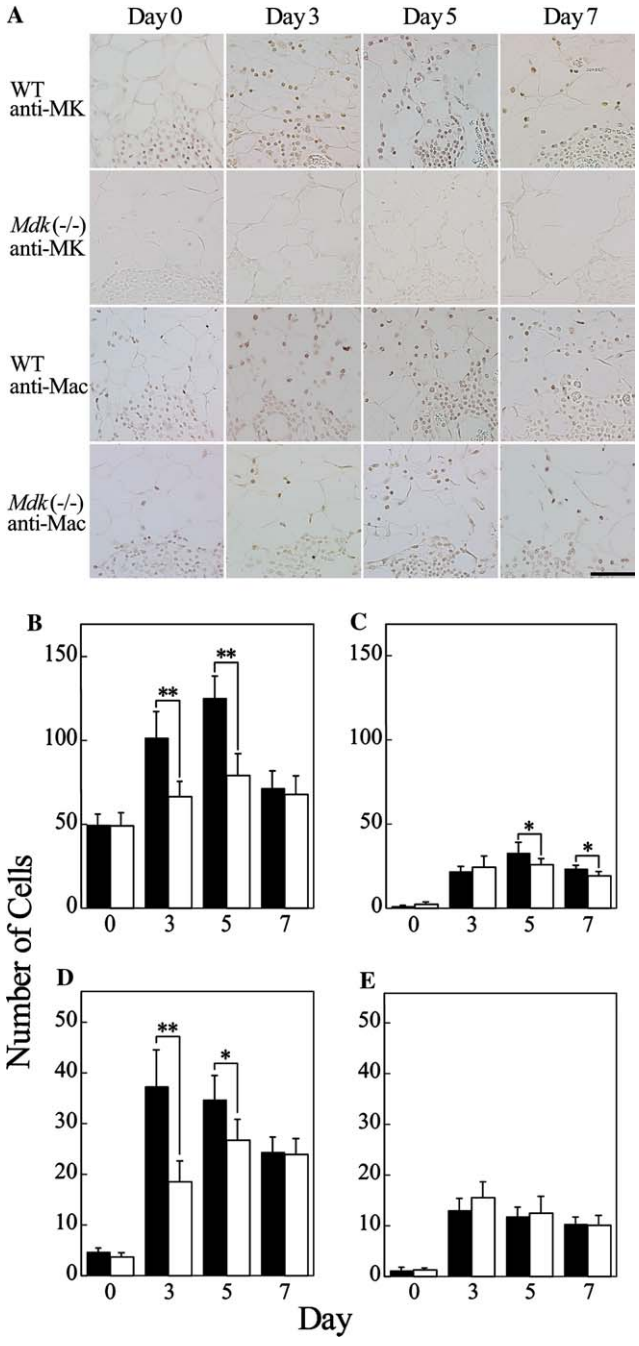

Fig. 4. Decreased migration of macrophages in the omentum of *Mdk* (−/−) mice. (A) Immunohistochemical staining of sections from the omentum is shown at different days after the wounding. Bar, 50 μm. (B–E) Quantitative estimation of decreased migration of inflammatory leukocytes in *Mdk* (−/−) mice. Numbers of macrophages (B,C) and neutrophils (D,E) present in the omentum (B,D) and the injured peritoneal wall (C,E) are shown. Closed bar, WT; open bar, *Mdk* (−/−). $^{*}p < 0.05$; $^{**}p < 0.01$. Statistical significance was evaluated with Student's *t* test.

Decreased migration of macrophages and neutrophils to the omentum in Mdk (−/−) mice

We observed that the number of macrophages in the omentum around the adhesive region peaked on Days 3 and 5 after the injury, indicating recruitment of the cells into these sites during adhesion (Fig. 4A). One of the sources of macrophages is milky spots in the omentum [30]. The migration was more marked in WT mice than *Mdk* (−/−) mice. Quantitative estimation confirmed the above observation (Fig. 4B). In terms of neutrophil migration to the omentum, we reached the same conclusion (Fig. 4D). Leukocyte migration to the injured peritoneal wall was only slightly different between WT and *Mdk* (−/−) mice (Figs. 4C and E).

Discussion

The present investigation demonstrated that MK is a key factor in the development of intraperitoneal adhesions. In *Mdk* (−/−) mice, the area of the omentum where adhesion occurred decreased to 8.3% of the value in WT mice, and the incidence of adhesion decreased from 90% to 20%. Such a marked decrease after cytokine depletion has not been reported previously. It was reported that intraperitoneal adhesions decreased about 50%, when the function of MCP-1 was inhibited by anti-MCP-1 [17]. The dramatic effect of MK depletion is probably due to the fact that MK promotes the migration of both neutrophils and macrophages directly and also indirectly through the induction of chemokines [24–26].

In *Mdk* (−/−) mice, neointima formation [24] and nephritis [26] upon ischemic injury are significantly suppressed with a concomitant decrease of inflammatory leukocyte recruitment as compared to WT mice. We consider that the following chain of events takes place in the case of adhesion. The injured peritoneal wall secretes factors that activate the omentum and trigger leukocyte recruitment. One of the sources of macrophages is milky spots within the omentum [30]. Recruitment of inflammatory leukocytes in the omentum is promoted by MK, which is present in blood vessels and is also secreted by macrophages. MK within macrophages can also contribute to their activation. After macrophage recruitment, MK secreted by them will further accelerate leukocyte recruitment. Recruited leukocytes may secrete factors that promote the adhesive activity of the omentum. The possibility that MK also directly stimulates the omentum cannot be completely excluded. We consider that in organ-to-organ adhesion, peritoneal macrophages play critical roles, and MK

contributes by promoting the migration and/or activation of macrophages. In this respect, it should be pointed out that the milky spots in the omentum are considered important sources of peritoneal macrophages [30].

The identification of MK as a key factor in the formation of intraperitoneal adhesions provides new insight into the mechanism behind the process and how to prevent them. In particular, therapeutic strategies to inhibit the action of MK or its biosynthesis would be useful in prevention. MK is strongly expressed during midgestation, while its expression in the adult is restricted to certain tissues and cells such as renal epithelial cells [31,32]. MK becomes generally overexpressed in inflammation and repair and also upon tumorigenesis [19,33–35]. Because of its mode of expression, MK is a suitable target in terms of few side effects. Anti-MK antibodies and MK antisense oligo DNA have already been developed and found effective in inhibiting the MK-dependent growth of tumor cells [28,36].

Equally important is a possible difference in the level of MK expression in the omentum between different patients. In the case where strong MK expression in the omentum is correlated with extensive intraperitoneal adhesions, it will become necessary to identify the promoter sequence required for the expression. Such information may lead to the identification of patients at risk of developing severe intraperitoneal adhesions based on the possible sequence difference in the MK promoter.

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