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Anti-metastatic Effect of Ginseng Saponins and its Molecular Mechanism

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Ginseng (the root of *Panax ginseng* C. A. MEYER, Araliaceae) has been used for traditional medicine in China, Korea, Japan and other Asian countries for the treatment of various diseases, including psychiatric and neurologic diseases as well as diabetes mellitus. So far, ginseng saponins (ginsenosides) have been regarded as the principal components responsible for the pharmacological activities of ginseng. Ginsenosides are glycosides containing an aglycone (protopanaxadiol or protopanaxatriol) with a dammarane skeleton and have been shown to possess various biological activities, including the enhancement of cholesterol biosynthesis, stimulation of serum protein synthesis, immunomodulatory effects and anti-inflammatory activity (Sakakibara *et al.*, 1975; Shibata *et al.*, 1976; Toda *et al.*, 1990; Scaglione *et al.*, 1990; Wu *et al.*, 1992). Several studies using ginsenosides have also reported antitumor effects, particularly the inhibition of tumor-induced angiogenesis (Sato *et al.*, 1994), tumor invasion and metastasis (Mochizuki *et al.*, 1995; Shinkai *et al.*, 1996), and the control of phenotypic expression and differentiation of tumor cells (Odashima *et al.*, 1985; Ota *et al.*, 1987). Previously, it was reported that protopanaxadiol-type and protopanaxatriol-type ginsenosides are metabolized by intestinal bacteria after oral administration to their final derivative 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol [referred to as M1 (Hasegawa *et al.*, 1996) or compound K (Kanaoka *et al.*, 1994; Karikuma *et al.*, 1991)] or 20(S)-protopanaxatriol [referred to as M4 (Hasegawa *et al.*, 1996)]. This made it unclear whether or not the expression of anti-metastatic effect by oral administration of ginsenosides can be induced by their metabolites.

We have recently reported that protopanaxadiol- or protopanaxatriol-type ginsenosides and their major metabolites M1 and M4 markedly inhibited lung metastasis of B16-BL6 melanoma cells when they were administered 5 times orally (Fig. 1) (Wakabayashi *et al.*, 1997a and 1997b).

In contrast, three consecutive i.v. administration of metabolite M1 and M4 after tumor inoculation resulted in a significant inhibition of lung metastasis, whereas ginsenosides Rb1, Rb2, Rc, Re and Rg_1 did not show any inhibitory effect (Fig. 2). These findings suggest that the expression of the *in vivo* anti-metastatic effect by oral administration of both types of ginsenosides was primarily based on their metabolite M1 and M4.

These results may be also supported by the finding that metabolites were detected in the serum from mice orally given ginsenosides, but ginsenosides were not detected by HPLC analysis. This pharmacokinetic study is in good agreement with previous reports on the low absorption rate of Rb1 from the intestines (Odani *et al.*, 1983; Tanizawa *et al.*, 1993) and high metabolic rate of Rb1 to M1 (Tanizawa *et al.*, 1993) in rat and human by using HPLC and enzyme-immunoassay methods (Hasegawa *et al.*, 1996; Kanaoka *et al.*, 1994). Moreover, it has also been noted that ginsenosides are hardly decomposed by gastric juice with the exception of slight oxygenation (Karikuma *et al.*, 1991). Therefore, our findings support the notion that ginsenosides may act as natural pro-drug which can be transformed to M1 by intestinal anaerobe(s) after oral administration and consequently induce *in vivo* anti-metastatic effect.

To investigate the incidence of intesinal bacteria possessing ginsenoside Rb1-hydrolyzing potential, hydrolyzing potential of intestinal bacteria, expressed as the transformation rate of Rb1 to metabolite M1, was carried out by using fecal specimens of mice. There were some correlations of the transformation rate of Rb1 to M1 in between mother mice and their litters, particularly, statistically significant between the groups of litters born from mothers with different rates of hydrolyzing potential. This suggests that the intestinal microflora of litter are primarily infected from mother. On the other hand, consecutive administration of ginseng extract to the mice with transformation rate from Rb1 to M1 of 25 ±11%, resulted in a significant increase in the transformation rate, as compared with untreated group. However, induction of Rb1-

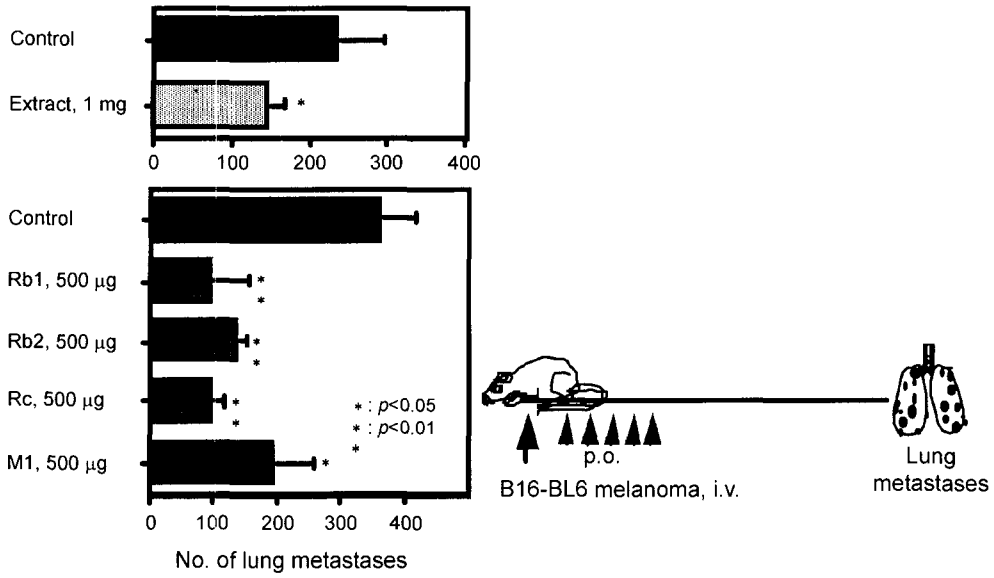

Fig. 1. Effect fo ginseng saponis on lung metastasis by i,v, injection of B-16 melanoma cells.

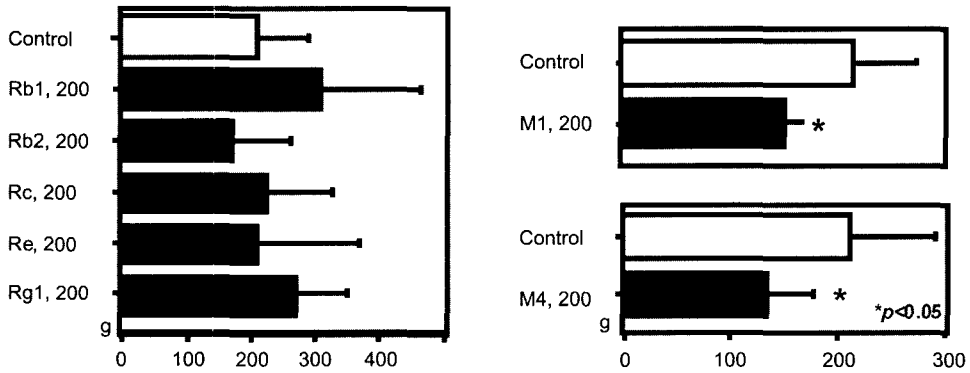

Fig. 2. Effect fo i.v. administration of ginseng saponins and their metabolites M1 and M4 on lung metastasis by i.v. injection of B-16-BL6 melanoma cells.

hydrolyzing potential by the administration of ginseng extract was hardly effective for the mice with hydrolyzing potential of less than 10%. For such mice, the inoculation of fecal microflora from mice with high hydrolyzing potential was also ineffective. Therefore, the location of the bacteria capable of hydrolyzing Rb1 on intestinal epithelium cells may be associated with genetic factors of hosts.

To examine the influence of Rb1-hydrolyzing potential on anti-metastatic efficacy, Rb1 was orally administered to two sets of mice with low and high hydrolyzing potential after s.c. inoculation of LLC tumor. A significant difference between active and inactive groups and also the tendency of a positive relationship between hydrolyzing potential and inhibition of lung metastasis were observed. These findings indicate that the transformation rate of Rb1 to its active metabolite M1 was dependent on Rb1-hydrolyzing potential of intestinal bacteria, which consequently affected the expression of anti-metastatic efficacy of orally administered Rb1 (Fig. 3). Thus, hydrolyzing potential of intestinal bacteria for crude drug or the formulation may be an important factor influencing the holistic pattern of symptoms and individual pathogenic alterations, so-called SHO, by which the diagnosis of disease state and the ways of treatment in Kampo are determined.

On the other hand, these ginsenosides hardly inhibited the invasion, migration and growth of tumor cells *in vitro*, whereas intestinal bacterial metabolites M1 and M4 showed the inhibitiory effects dose-dependently (Fig. 4) (Wakabayashi *et al.*, 1997a and 1997b).

These findings indicate that M1 produced in the serum

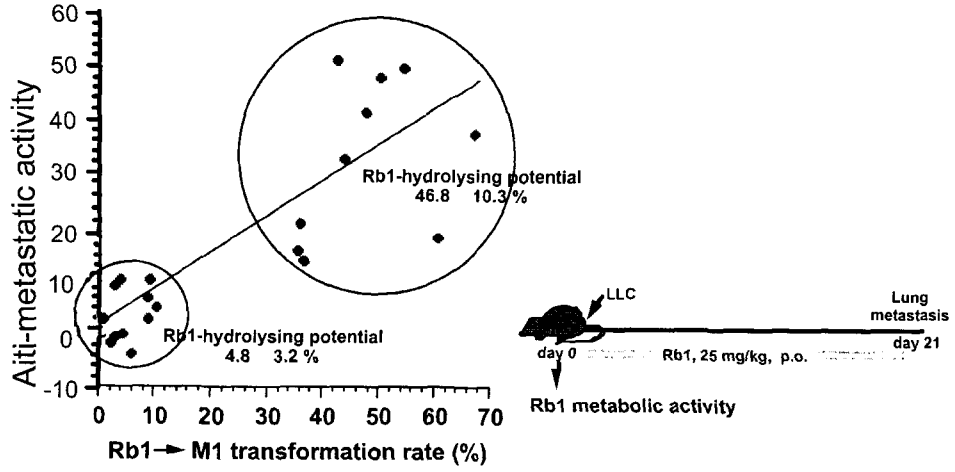

Fig. 3.

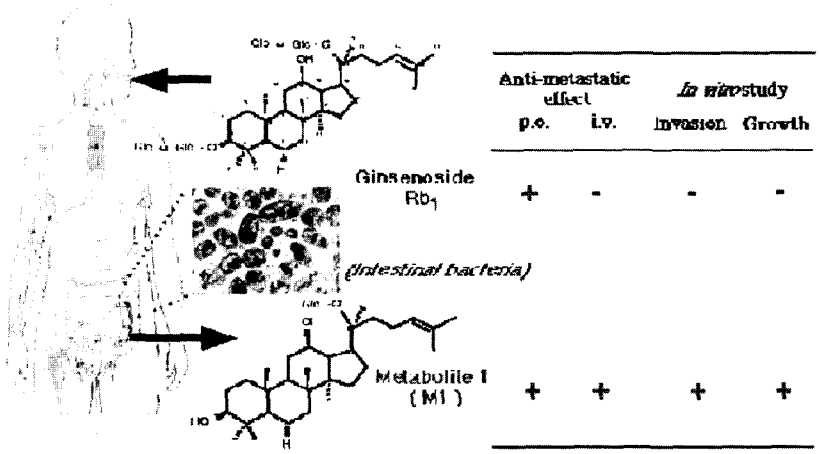

Fig. 4.

after the oral administration of ginsenoside Rb1 may induce the *in vivo* anti-metastatic effect partly through the inhibition of tumor invasion, migration and growth of tumor cells. Even if direct addition of ginsenosides into the culture *in vitro*, which is referred to as FURIKAKE (in Japanese) assay, was effective at inhibiting tumor cell invasion and proliferation, these results should not be apparently accepted for the explanation of *in vivo* efficacy by oral administration of ginsenosides.

However, the detail of how M1 affects the growth of tumor cells has not been clear. Co-incubation of tumor cells with M1 at concentrations ranging from 5 to 40 μM resulted in a time- and concentration-dependent inhibition of tumor cell proliferation, with accompanying morphological changes (spindle-shape) at the concentration of 20 μM (Wakabayashi *et al.*, 1998). In addition, M1 at a concentration of 40 μM caused the cytotoxic response in tumor cells at an earlier time period (within 24 h) in the culture (Fig. 5). Since the swelling-shape morphology of tumor cells is considered to be an apoptotic character, the cell death by treatment with M1 (40 μM) was due to the induction of apoptosis. The ladder fragmentation of the extracted DNA and swollen-round morphology indicated that the cell death was caused by apoptosis. In contrast, the incubation with ginsenoside-Rb_1 (40 μM) did not affect the morphology of tumor cells or cell proliferation (Wakabayashi *et al.*, 1997a and 1998).

Although the molecular events that drive the apoptotic signaling pathway are not entirely clear, some apoptosis-related proteins such as cyclin D1, c-Myc or cyclin-dependent kinase (CDK) inhibitors have been reported to be associated with cell division and proliferation (Fukumoto *et al.*, 1997; Rogatsky *et al.*, 1997; Vlach *et al.*, 1996). Therefore, in order to clarify the mechanism of M1-induced apoptosis, we investigated the effect of M1 on the expression of the apoptosis-related proteins, p21,

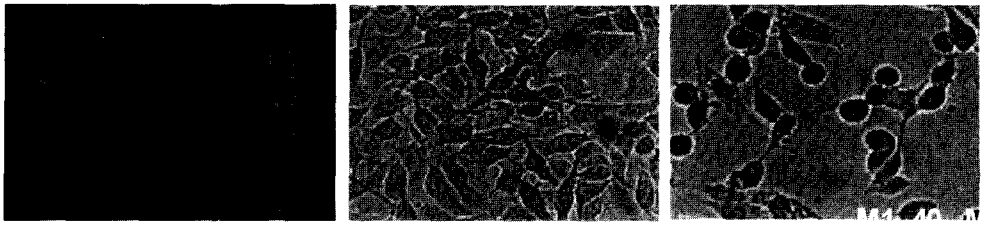

Fig. 5. M1-induced DNA fragmentation of B-16-BL6 cells and the cell morphology.

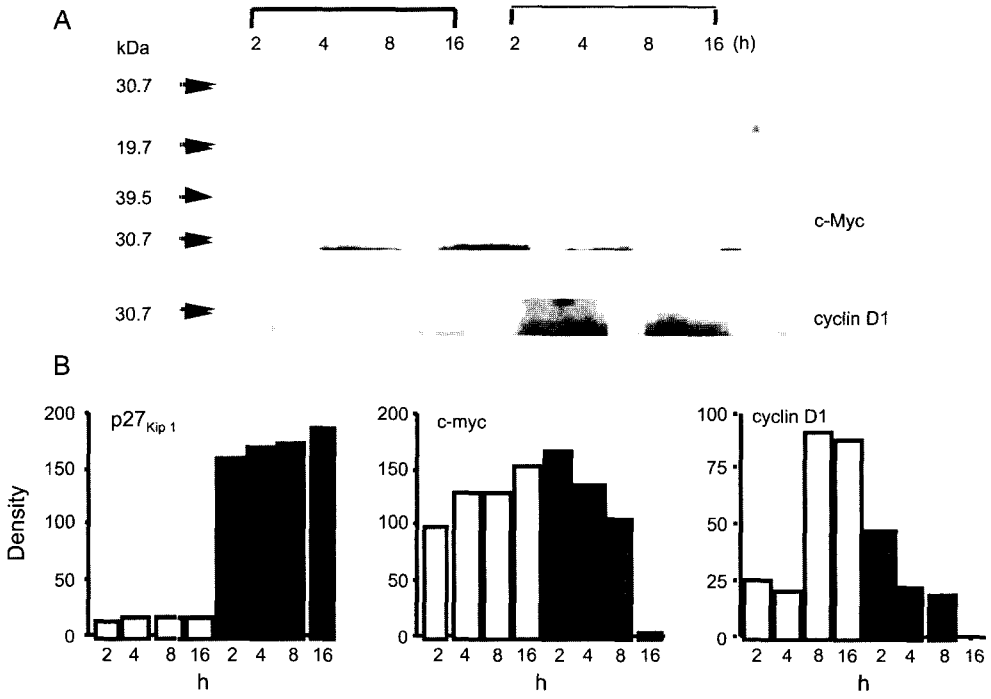

Fig. 6. Western blot analysis of p27Kipl, c-Myc and cyclin D1 in B16-BL6 cells trated with M1.

$p27^{Kipl}$, c-Myc and cyclin D1. M1 treatment (40 μM) markedly increased the expression of $p27^{Kipl}$ as compared with the untreated control (Fig. 6).

No expression of the other CDK inhibitor, p21 was detected in B16-BL6 cells in this experiment (data not shown). The up-regulation of $p27^{Kipl}$, which is known to inhibit the CDK activity, was observed during the apoptotic process caused by anti-cancer agents including etoposide and camptotecin. On the other hand, a proto-oncogene product c-Myc as well as cyclin D1 have been reported to be overexpressed in the proliferative phase of various types of tumor cells (Fukumoto *et al.*, 1997; Rogatsky *et al.*, 1997; Vlach *et al.*, 1996). The expression of c-Myc and cyclin D1 was down-regulated by M1 treatment in a time-dependent manner. Thus, M1 might cause the cell-cycle arrest in tumor cells through the up/down-regulation of these cell-growth related molecules, and consequently induce apoptosis.

It has been reported that various molecules such as Bcl-2 (an inhibitor of apoptotic cell death), Bax (promotion of apoptosis by antagonizing the function of Bcl-2) and caspases (interleukin-1 converting enzymes to trigger the execution of cell death) are involved in positively or negatively regulating apoptosis signaling (Story and Kodym, 1998; White, 1996; Vaux and Strasser, 1996). Recent studies have proposed some signaling pathways for apoptosis mediated by different regulatory molecules (Story and Kodym, 1998; Evans *et al.*, 1995). Therefore, the possibility that M1 inhibits or promotes these apoptosis-related molecules will be needed to be examined.

We also examined the intra-cellular distribution of M1 after the incubation of tumor cells with dansyl M1. The fluorescent signal of dansyl M1 was detected in the cytosol and nuclei 15-min after incubation, and thereafter was observed predominantly in the nuclei. These findings suggest that the apoptotic cell death is induced by intra-cel-

lular M1 through the transcriptional regulation of several cell-growth associated proteins. Since M1 has a steroid-like chemical structure, it may interact with some intracellular receptors including a steroid receptor, which are known to be involved in the rapid regulation of nuclear proto-oncogene transcription (Schuchard *et al.*, 1993). The regulatory mechanisms of M1 at the transcriptional level will be needed to investigate in detail.

In summary, we demonstrated that a metabolite of ginseng protopanaxadiol saponin (M1), with anti-metastatic property, inhibited the proliferation of tumor cells in a time- and concentration-dependent manner, and in addition induced apoptotic cell death. The induction of apoptosis by M1 involved the up-regulation of the CDK-inhibtor $p27^{Kip1}$ as well as the down-regulation of c-Myc and cyclin D1 (Fig. 7). The nucleosomal distribution of M1 suggests that the modification of these molecules is induced by transcriptional regulation.

The induction of tumor angiogenesis is believed to reflect a balance between positive and negative regulatory factors (Folkman and Shing, 1992). Some angiogenesis-related molecules, such as platelet derived growth factor (Battegay *et al.*, 1994), vascular endotherial cell growth factor (Warren *et al.*, 1995) and fibrobrast growth factor (Esch *et al.*, 1985) play an important role in positively regulating the formation of tumor angiogenesis. On the contrary, angiostatin (O'Reilly *et al.*, 1994), thrombospondin (Weinstat-Saslow *et al.*, 1994), palatelet factor 4 (Kolber *et al.*, 1995), interferon (Arenberg *et al.*, 1996), endostatin (O'Reilly *et al.*, 1997), interferon (Dinney *et al.*, 1998) and nitric oxide (Xie and Fidler, 1998) inhibit tumor angiogenesis. Therefore, we examined whether or not M1 can affect tumor-induced angiogenesis.

Recently, Tanigawa *et al.* (1997) reported that hepatic

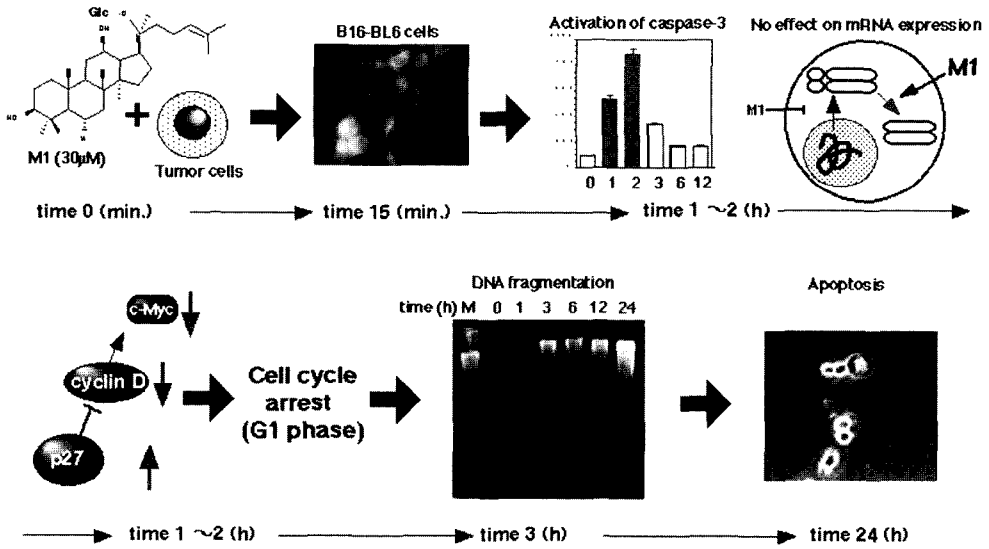

Fig. 7. Mechanism of M1-induced growth inhibition of tumor cells.

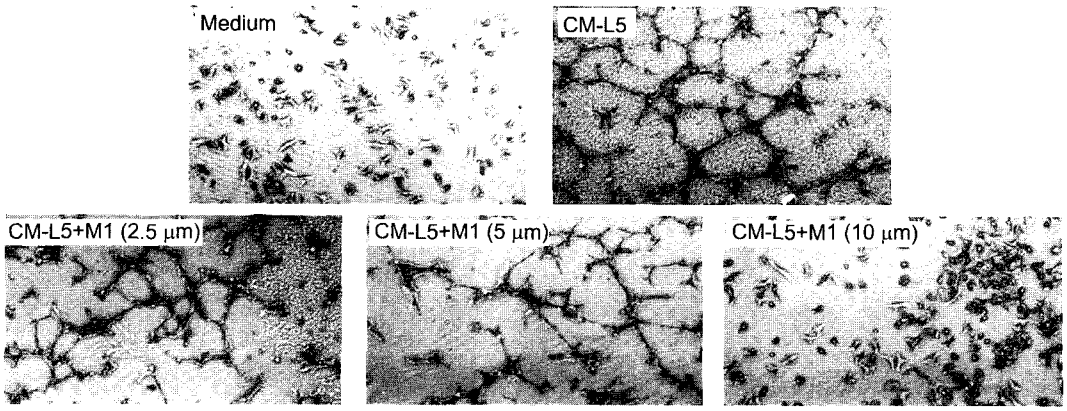

Fig. 8.

sinusoidal endothelial (HSE) cells are associated with tumor-induced angiogenesis in the liver. The proliferation and tube formation of HSE cells are stimulated by vascular endothelial growth factor in conditioned medium of colon 26-L5 cells (CM-L5) (Murakami *et al.*, 1999). Thus, tumor-induced angiogenesis was assessed following tube formation of HSE cells formed on Matrigel-coated plates.

The incubation of HSE cells with 50% CM-L5 caused tube-like structures of HSE cells within 4 h (Fig. 8). Addition of CM-L5 obtained from the cultures of colon 26-L5 cells with M1 (2.5, 5 or 10 μM) resulted in a concentration-dependent inhibition of the tube formation of HSE cells. The incubation of colon 26-L5 cells with M1 at 0~10 μM for 24 h. did not affect the growth of HSE cells (data not shown). Moreover, the incubation of HSE cells with M1 at 0~20 μM for 72 h. did not affect the growth of HSE cells, nor the formation of tube-like structures (data not shown).

The incubation with CM-L5 markedly enhanced the migration of HSE cells as compared with the untreated control. CM-L5 obtained from the culture of colon 26-L5 cells with M1 resulted in a concentration-dependent decrease of CM-L5-enhanced migration. However, M1 at the concentrations used in this study did not directly affect the inhibition of HSE cell migration (date not shown).

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