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Depolymerized Products of λ-Carrageenan as a Potent Angiogenesis Inhibitor

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Since angiogenesis is involved in initiating and promoting several diseases such as cancer and cardiovascular events, this study was designed to evaluate the anti-angiogenesis of low-molecular-weight (LMW), highly sulfated λ-carrageenan oligosaccharides (λ-CO) obtained by carrageenan depolymerization, by CAM (chick chorioallantoic membrane) model and human umbilical vein endothelial cells (HUVECs). Significant inhibition of vessel growth was observed at 200 μg/pellet. A histochemistry assay also revealed a decrease of capillary plexus and connective tissue in λ-CO treated samples. λ-CO inhibited the viability of cells at the high concentration of 1 mg/mL, whereas it affected the cell survival slightly (>95%) at a low concentration (<250 μg/mL), and HUVEC is the most sensitive to λ-CO among three kinds of cells. Furthermore, the inhibitory action of λ-CO was also observed in the endothelial cell invasion and migration at relatively low concentration (150−300 μg/mL), through down-regulation of intracellular matrix metalloproteinases (MMP-2) expression on endothelial cells. Taken together, these findings demonstrate that λ-CO is a potential angiogenesis inhibitor with combined effects of inhibiting invasion, migration, and proliferation.

KEYWORDS: Angiogenesis; λ-carrageenan; oligosaccharides; chick chorioallantoic membrane; human umbilical vein endothelial cells

INTRODUCTION

Sulfated saccharides are a class of compounds presenting numerous negatively charged sulfate groups that vary in position in their saccharide chains. Sulfated saccharides have been proved to show a wide range of biological activities important to human health, for example, antiviral, antitumoral, anti-inflammatory, and anticoagulant (e.g., heparin derivatives (*1*, *2*), laminarin sulfate (*3*), and chitin derivatives (*4*)). In recent years, several classes of sulfated poly- or oligosaccharides have been demonstrated to show anti-angiogenesis activity by inhibiting heparanase or blocking the binding of growth factors with their receptors. The compounds tested included pentosan polysulfate (*5*), degraded heparin, suramin (*6*), and also λ-carrageenans (*7*). The most widely known and therapeutically used sulfated saccharide is endogenous heparin, which is one of the most negatively charged molecules in nature (*8*). Over the past few decades, discovery of the sequences in heparin has been invaluable toward understanding the biological activities of heparin, so the potential therapeutic usage of heparin has been fully recognized (*9*). But because of its endogenetic property, heparin as a therapeutic agent will suffer from molecular unspecificity. Therefore, much of the effort aiming at heparin-mimicking anionic sulfated saccharides coming from natural plants or animals has been required to exploit the numerous potential therapeutic applications (*10*).

Carrageenans, a family of sulfated polysaccharides isolated from marine red algae, are widely used as food additives, such as emulsifiers, stabilizers, or thickeners. The carrageenan family shares the same backbone structure, which consists of a repeating disaccharide backbone of alternating 3-linked β-D-galactopyranose (G) and 4-linked α-D-galactopyranose (D), with 3,6-anhydrogalactose residues commonly present. Depending on the number and position of anionic O-sulfo (sulfate) groups, several types of carrageenans can be recognized. For example, the three most commercially exploited carrageenans, namely kappa- (κ, DA-G4S), iota- (ι, DA2S-G4S), and lambda- (λ, D2S, 6S-G2S) carrageenans, differ by the presence of one, two, and three sulfate ester groups per repeating disaccharide unit, respectively (*11*−*13*). They have been found to show biological activities such as anticoagulant activity (*14*), antitumor activity, antagonistic activity against basic fibroblast growth factor or platelet-derived growth factor (*15*), and antiviral activity (*16*, *17*). However, based on recent studies, the type and extent

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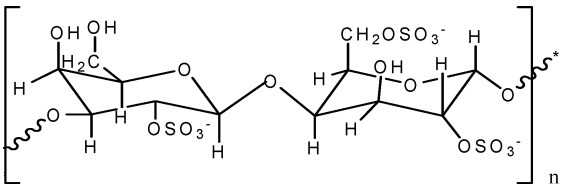

Figure 1. Structure of λ-carrageenan oligosaccharides.

of activities will vary depending on the content and position of the sulfate on the molecule. Generally, the sulfated moieties in saccharides are believed to play an important role in manifestation of beneficial bioactivity (*18*, *19*). In these carrageenans, λ-carrageenan contains approximately a 35% ester sulfate content and almost no content of 3,6-AG, possessing the maximum degree of sulfate substitution in the carrageenan family. So it is the most hopeful molecule to be developed into an antiangiogenesis agent. However, compared with sulfated polysaccharides, sulfated oligosaccharides express more of an advantage because they are structurally more homogeneous and exhibit less toxicity due to reduced anticoagulant activity and likely ease of excretion In addition, oral delivery may be more feasible. Moreover, many activities can be potentiated by depolymerization, such as anticoagulant and antiviral activity (*20*), which suggested that we may increase the bioactivity of λ-carrageenan by changing its molecular weight. There are also a few reports on antitumor activities of low-molecular-weight (LMW) λ-carrageenan, such as activities toward mammary adenocarcinoma, Meth-A tumor, and Ehrlich ascites cells (*21*, *22*). But except for the immunomodulation effect, no other mechanism has been reported to explain its antitumor effect (*23*). Since some LMW oligosaccharides with heparin fragment-like structures may act as angiogenesis inhibitors (*10*), it is possible that heparin-like λ-carrageenan oligosaccharides from red algae might be found to be low-cost, broad-spectrum anti-angiogenesis agents. This would be interesting, particularly if they served to palliate or even hopefully cure cancer.

For this purpose, we initially examine the effect of λ-CO with about 3350 molecular weight on angiogenesis by the CAM (chick chorioallantoic membrane) model. In an effort to elucidate the mechanisms which mediate the anti-angiogenic process of this oligosaccharides, the viability, migration, and invasion activity of human umbilical vein endothelial cells (HUVECs) were also monitored. Moreover, considering the good relationship between endothelial cells' invasion and MMP-2, the expression of MMP-2 was examined.

MATERIALS AND METHODS

Materials. The λ-carrageenan oligosaccharides used in this study were obtained by solid acid (cationic exchange resin) mediated hydrolysis: 1% λ-carrageenan was suspended in water containing 20% cationic exchange resin, and the solution was held at 90 °C for 6 h. The hydrolysate was neutralized, desalted, freeze-dried, and stored at 4 °C prior to experiments. The structures of the oligosaccharides were identified as a mixture of oligosaccharides with a basic biose unit of galactose−galactose substituted by three sulfate ester groups (**Figure 1**), with average molecular weight of 3350, and the degree of polymerization of these oligosaccharides mainly ranges from 4 to 12, as detected by detecting thin-layer chromatography.

CAM Assay. To investigate the *in vitro* angiogenic activity, the chick chorioallantoic membrane assay with a modified procedure based on Kirchner et al. (*24*) was carried out. Briefly, the surfaces of 5-day-old postfertilization chick eggs (commercial White Leghorn, obtained from a local hatchery) were sterilized and incubated blunt end up in an egg incubator at 60% relative humidity for 3 days. Eggs were cracked, the egg contents were allowed to flow into a sterile cup, and the cup was covered with a sterile plastic tissue culture dish cover with a small opening to allow for air exchange and maintained in incubator until the CAM grew to maturity and was entirely visible. During the next 2−3 days, extensive capillary formation took place. A sponge was then placed on the exposed CAM. Aliquots (5 μL) of solution with or without oligosaccharides were added onto the sponge. After 48 h of incubation, the area around the loaded disk was cut and photographed with a digital camera, and the vascular density was estimated from the number of newly formed vessels counted by three observers in a double-blind manner. Assays for each test sample were carried out using 8 eggs. After imaging, CAMs were fixed in 10% formaldehyde solution and processed for histological examination. The tissues were then embedded in paraffin wax, sectioned at 5 μm thickness, mounted on slides, and stained with hemeatoxylin-eosin (H&E) for routine light microscopy. The slides were investigated for subtle changes in CAM capillary plexus formation.

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were obtained from the ATCC (Rockville, MD). Human liver cancer cell Bel-7402 and normal human hepatocyte L-02 were obtained from CCTCC (Wuhan, China). The three cell lines were cultured in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum and 100 U of penicillin G and 100 μg/mL of streptomycin. HUVECs need to be supplied with an additional 75 μg/mL of endothelial cell growth factor (ECGF) (Sigma). The cultures were maintained at 37 °C in a 5% CO_2/95% air atmosphere.

Cell Viability Assay. The MTT assay was used for the quantitative determination of cellular survival and growth (*25*). HUVECs were seeded and incubated at indicated concentrations of λ-CO in the MEM medium for 24 or 48 h. The spectrophotometrical absorbance of the samples was measured at a wavelength of 492 nm. The data were expressed as percentage of the control group. The results of three experiments in triplicate are expressed in percentage of control.

Capillary Tube Formation on a Matrigel. The formation of capillary tubelike structures by HUVE cells was analyzed on 96-well cell culture plates coated with an extracellular membrane matrix (Matrigel; BD Biosciences, Bedford, MA) (*26*). Matrigel was thawed at 4 °C overnight and 1:1 diluted with serum-free medium. Using precooled plates, 25 μL/well of matrigel was distributed and allowed to solidify at 37 °C for at least 30 min. Cells were seeded on the polymerized matrigel (3×10^4 cells/well). The plate was incubated at 37 °C, and tube formation was observed under a phase-contrast microscope. Digital pictures were taken after 24 h with a camera.

Cell Invasion Assay. HUVECs' invasion was evaluated using 24-well transwell cell culture chambers with 8 μm pore polycarbonate filter inserts. An aliquot of 40 μL of matrigel was added into each filter insert and incubated at 37 °C for 30 min. Cultured HUVECs were trypsinized and suspended in 0.1% BSA/MEM at a concentration of 2×10^5/mL. A total of 400 μL of cell suspension was applied to coated insert filters, and 600 μL of 20% BSA/MEM was added to the lower chamber. Various concentrations of λ-CO were added to the upper compartment. The chamber was incubated for 24 h to allow cell migration. The insert was removed, and the membrane was washed with 0.1 M PBS. No migrated cells on the upper side of filters were scraped with cotton swabs; migrated cells on the lower side of chambers were fixed and stained with hematoxylin. The membrane was examined under a microscope. Migration was quantified by measuring the stained cells in three random areas per membrane (*27*).

Wound Assay. Confluent HUVE cells grown in 24-well plates were mechanically wounded by scraping away one side of the cells to obtain a denuded area. After scraping, cells were rinsed twice with medium to remove wound-derived loose and dislodged cells. Wounded cells were further cultured in medium for the next 24 and 48 h with or without the indicated concentration of λ-CO. After the wounding, movement of cells into the denuded area was recorded with an inverted Olympus microscope immediately (time zero controls) as well as 24 and 48 h later. Migration of cells was quantified using an image analysis of three fields of view of the denuded area examined at random.

MMP-2 Expression Assay. HUVE cells were plated on glass coverslips in 24-well plates at a density of 5×10^5 cells/mL and allowed to grow to the desired confluency. Subsequently, cells were incubated for 24 h with or without λ-CO and the coverslips were washed carefully

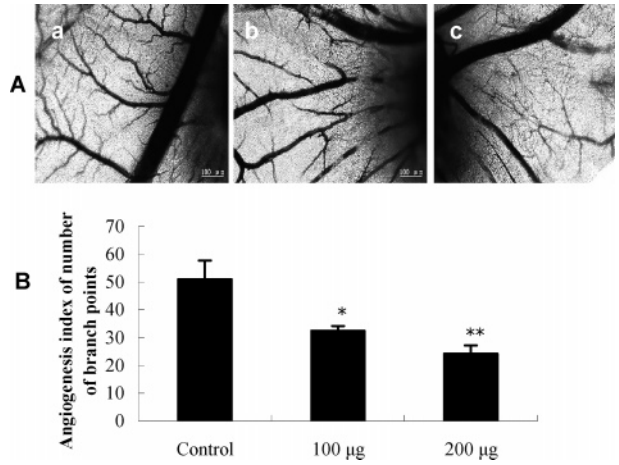

Figure 2. (**A**) Angiogenesis in the chick chorioallantoic membrane (CAM). It demonstrates an arborizing network of vessels. Development of vasculature in CAMs is inhibited by λ-CO. (a) Vehicles alone. (b) 100 μg/pellet. (c) 200 μg/pellet. Solutions of oligosaccharides (5 μL) were added on day 8. Photographs represent CAM development on day 10. The dark area is the position where the sponge is placed. (**B**) Number of vessel cross sections per 0.6 mm^2 CAM. Oligosaccharides were implanted via a gelatin sponge. On day 10, the sponge and the immediate surrounding CAM tissues were excised, processed, and fixed, and their blood vessel cross sections ($n = 8$ in each group) were counted in a blind manner. The asterisk indicates a statistical difference ($P < 0.05$).

with PBS and fixed in methanol for 5 min. Immunocytochemical staining for MMP-2 was performed with anti-MMP-2 antibody (rat). The three-step immunoperoxidase method using an Ultra sensitive S-P kit (MaiXin Biotechnology, Fuzhou, China) was carried out according to the instructions provided by the manufacture. 3,3-*p*-Diaminobenzidine (DAB) was used as substrate. Then HUVECs were counterstained with hematoxylin. A negative control was performed by the incubation of PBS in substitution for the primary antibody. Positive immunostaining, which appeared as a brown color, was visualized under a microscope. The protein levels were quantified as integrated optical density (IOD) values with an image analyzer (Image-pro Plus software).

Statistical Analyses. All data are presented as mean ± standard deviation (SD) and are analyzed using the Student's *t*-test for significant difference. Statistical significance was defined as $P < 0.05$.

RESULTS

Evaluation of Angiogenesis by the CAM Model. The CAM assay is an important model of microvessel formation (*28*). The anti-angiogenic activities of λ-CO were investigated using this model. After administration of λ-CO and inoculation for 48 h, the change in blood vessel density was assessed in the area surrounding the sponge placement. **Figure 2** represents digitized images of representative CAMs treated for 48 h with vehicle (PBS) or λ-CO (100 or 200 μg/egg). The amount of branch vessels, particularly smaller vessels, was significantly decreased, and the decline was relevant to the increase in amount of oligosaccharides in that solution. Capillary quantification indicated that CAMs implanted with a gelatin sponge seeded with 200 μg/egg of oligosaccharides showed a significant decrease ($P < 0.01$) of more than 50% in vessel number inside the sponge environment when compared to egg treated with vector alone.

Examinations of histological sections of day 10 CAMs revealed a more pronounced presence of microvascular infiltrates within the membranes. The ectoderm and endoderm of control and treated CAMs were almost similar. In the control group, extensive capillary plexus and blood vessel formation (**Figure 3a**) were seen. Application of λ-CO caused a decrease in the

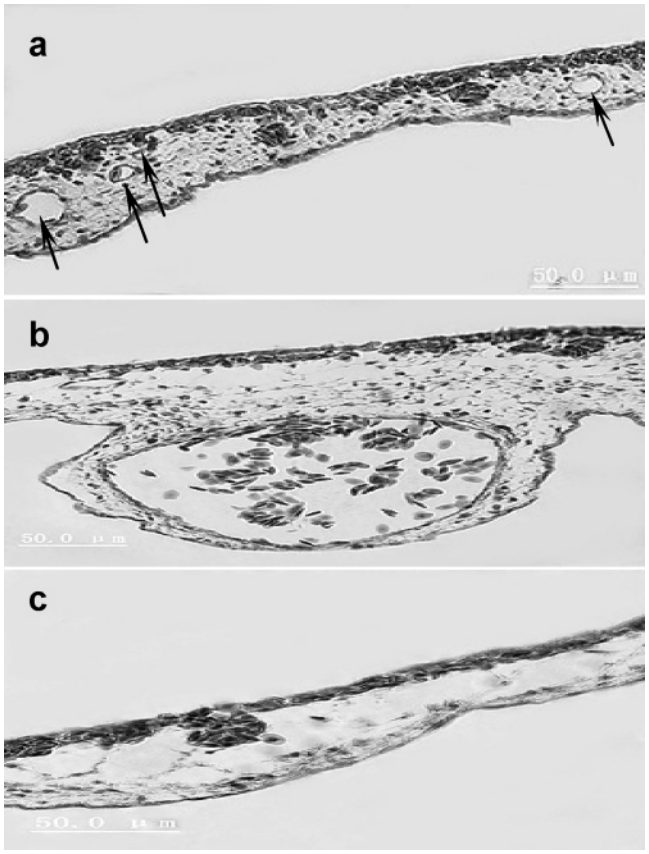

Figure 3. Histological view of day 10 mature chick CAMs implanted with oligosaccharides/sponge matrix (H&E stain, ×100 mag). (**a**) Control. The membrane shows a normal distribution and density of blood vessels and capillary plexuses (arrow). (**b**) 100 μg/mL λ-CO. (**c**) 200 μg/mL λ-CO.

amount and size of capillary plexus formation. The most changes were observed in the 200 μg/egg treated group, which contained scanty blood vessels and few fibroblasts and appeared to have less extracellular matrix than the mesoderm of control CAMs (**Figure 3c**).

Effect of λ-CO on HUVE Cells. In order to explore the acting mode of λ-CO on the CAM model, several crucial steps involved in the angiogenesis process were examined, including cell survival of endothelial cells, capillary tube formation, invasion, and migration. Human umbilical vein endothelial cells were used for these studies. First, we performed MTT assays to assess the effect of λ-CO on endothelial cell viability and to determine the proper oligosaccharide dose and treatment time for the following experiments. We treated HUVECs with various dosages of λ-CO for 24−48 h. The subsequent MTT assay showed that the significant and dose dependent inhibitory effect on HUVECs growth caused by λ-CO was observed at 24 h and persisted for 48 h (**Figure 4**). A high concentration of this oligosaccharide revealed an inhibit effect on cell survival, while, at low concentration (<250 μg/mL), carrageenan showed almost no effect. The viability of HUVECs was inhibited less than 5% at 125 μg/mL. To demonstrate whether λ-CO specifically inhibits the growth of endothelial cells, **Figure 5** gives an oligosaccharides dose response curve for growth-inhibitory potency against HUVECs, human liver cancer cell Bel-7402, and normal human hepatocyte L-02. All cell types were treated with 0.125−1 mg/mL of λ-CO and compared to vesicle treated control cells. At concentrations above 125 μg/mL, a dose-dependent inhibition of cell growth was observed in all cell types. However, within the tested concentration (125 μg/mL to 1 mg/mL), L-02 and Bel-7402 are apparently insensitive to the

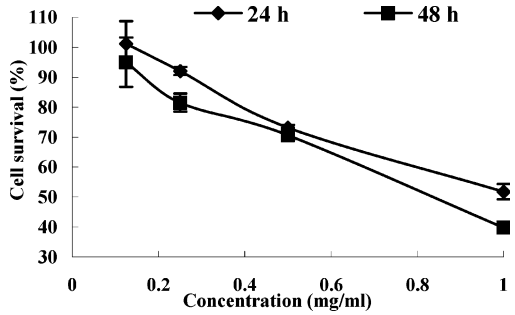

Figure 4. Quantification of inhibitory effects of λ-CO on HUVECs. HUVECs were plated in 96-well plates, allowed to attach for 24 h, and then treated with different concentrations of λ-CO for 24 and 48 h. Cell viability was determined by MTT assay. Data represent the average (±SD) of three experiments.

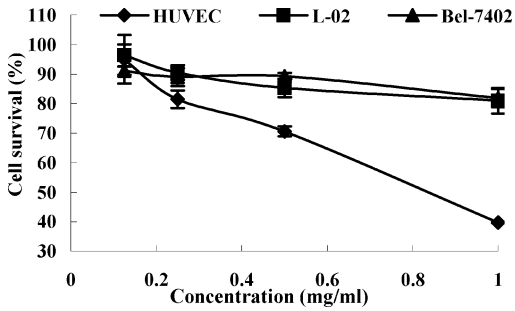

Figure 5. Influence of λ-CO on the cell survival of three cell lines. HUVECs, Bel-7402, and L-02 were plated in 96-well plates, allowed to attach for 24 h, and then treated with different concentrations of λ-CO for 48 h, respectively. Cell viability was determined by the MTT assay. Data represent the average (±SD) of three experiments.

presence of λ-CO. At 1 mg/mL, the survival ratio of these two cell lines was more than 80%. HUVECs were the most sensitive to λ-CO in this test panel and can be inhibited 60.23% at 1 mg/mL. Normal human hepatocyte L-02 can be inhibited only 19.01% at 1 mg/mL.

A capillary tube formation assay was performed to test the anti-angiogenic effect in a cellular system. The matrigel assay condition supported differentiation of untreated HUVECs into an extensive and complete network of capillary-like structures (**Figure 6a**). The capillary tubelike network was slightly inhibited at a lower dose of λ-CO (100 μM); however, treatment with higher doses of this oligosaccharide resulted in some fragments of unconnected tubes (200 μM, **Figure 6c**) as compared to the controls.

As endothelial cell invasion is a critical and initiating event in the angiogenesis, the ability of HUVECs to penetrate the reconstituted basement membrane matrigel was assessed. We counted the number of invaded cells 24 h after cell seeding and found that λ-CO efficiently suppressed the HUVECs invasion through the matrigel-coated filter (**Figure 7A**) in a concentration-dependent inhibitory manner. It could achieve a 55.56% inhibition ratio at 200 μg/mL (**Figure 7B**). From these data, we could speculate that λ-CO could inhibit angiogenesis by interrupting cellular invasion.

To investigate whether λ-CO regulates endothelial cellular migration, we looked at the ability of λ-CO to inhibit HUVEC migration using a wound migration assay. Confluently grown HUVECs were wounded, and the migrated cells were counted after 24 and 48 h. We observed that endothelial cells migrated more rapidly in response to control cells compared to λ-CO treated cells (**Figure 8**). In fact, there was an approximately 40−50% difference in cell migration between these two groups (**Figure 8B**). In the control group, wounded HUVECs migrated to confluence within 48 h, whereas cultures treated with oligosaccharides could not reach confluence even after 48 h. These data suggested that λ-CO had a profound impact on endothelial cells' migration.

Considering the gelatinase plays a key role during migration, invasion, and metastasizing of malignant cells (*29*), we analyzed the effect of λ-CO on the expression of MMP-2 to evaluate if the anti-invasive and antimigration effects of λ-CO were mediated by inhibiting activity of gelatinolytic MMPs. MMP-2 positive cells were distinguished by the brown-stained cytoplasm. The endothelial cells in the control group showed extensive expression of MMP-2. λ-CO down-regulated the expression of MMP-2 24 h after treatment; the staining of MMP-2 in experimental groups was weaker (**Figure 9A**). The MMP-2 protein level (IOD values) in the group treated with 120 μg/mL significantly reduced by about 76.02% ($P < 0.01$) (**Figure 9B**). These results suggested λ-CO has inhibitory effects on MMP-2 expression.

DISCUSSION

According to previous results, molecular weight and sulfated moieties are two critical factors to affect the anti-angiogenesis or antimetastatic activity of sulfate saccharides. Therefore, some sulfated oligosaccharides have been designed and synthesized aiming at enhancing the inhibition of tumor metastasis, arteriosclerosis, and angiogenesis (*1*, *3*). However, some difficulties still exist to develop heparin mimetic compounds, because these endogenous molecules always carry out multiple functions and are difficult to synthesize. Thus, low-molecular-weight sulfate oligosaccharides with high specificity that are easy to prepare are still needed. λ-Carrageenan, a polysulfated saccharide isolated and derived from the cell wall of seaweed containing extensive O-sulfation, has long been reported to exhibit an inhibitory effect on heparanase and to decrease metastasis formation and invasion (*30*, *31*). But to our knowledge, this is the first report about the anti-angiogenetic activity of depolymerized λ-carrageenan.

In the present study, our purpose was to investigate whether these low-molecular-weight, high-sulfate carrageenans can mimic the inhibitory effect of lots of sulfated saccharides on angiogenesis. The CAM model was used to assess the angiogenic ability because the vascular network on CAM could be displayed perfectly and multiple grafts could be analyzed concurrently (*32*, *33*). Actually, in order to obtain a large amount of anti-angiogenetic compound, we screened a series of red algae derived oligosaccharides, including κ-, ι-, and λ-carrageenan, with different molecular weights by this model. According to the CAM determinations, we found the most effective carrageenan is the λ-type. λ-Carrageenan oligosaccharides with average $M_w < 7000$ possessed anti-angiogenic ability, whereas κ- or ι-carrageenan oligosaccharides with any M_w or λ-carrageenan oligosaccharides with $M_w > 7000$ expressed no inhibitory activity. For other sulfated carbohydrates such as the heparins, it was stated that a minimum chain length of heparin fragments is required to demonstrate any anti-angiogenic effect (*34*). As in the case of heparin fragments, for the anti-angiogenic effect of the sulfated galacto-oligosaccharides, a minimum chain length is also required. Käsbauer et al. stated that DP 3-4 showed no or a weak activity, but at a chain length of five galactose units and higher, there is a pronounced anti-angiogenic effect (*35*). In our results, λ-carrageenan saccharides with low DPs show anti-angiogenic activity, especially the oligosaccharides with M_w around 3000, and this result is similar to others' conclusions.

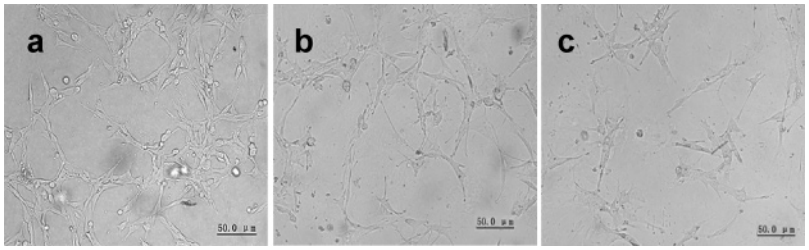

Figure 6. Influence of λ-CO on tube formations of HUVECs (×200 mag): (**a**) control; (**b**) treatment with 100 μg/mL λ-CO; (**c**) 200 μg/mL of λ-CO. HUVECs were grown on 96-well plates precoated with matrigel and λ-CO. The endothelial morphological changes were captured through a microscope and photographed.

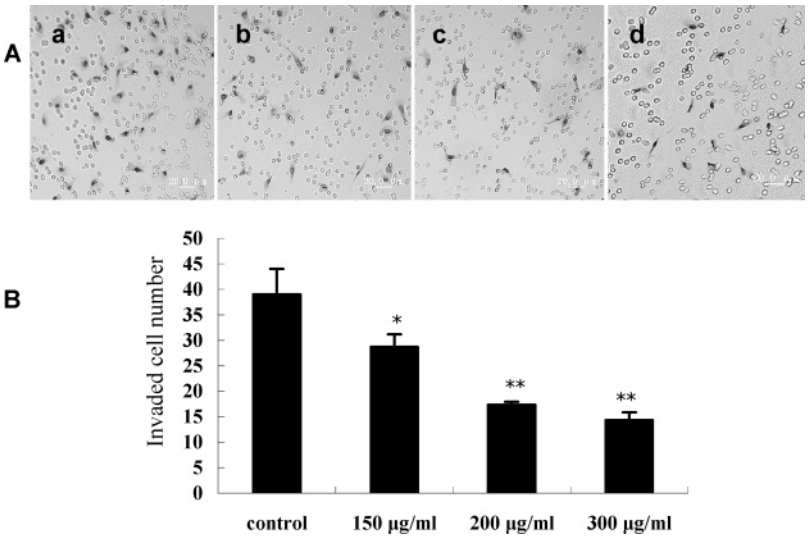

Figure 7. (**A**) Effect of λ-CO on HUVECs invasion by photos. (a) control; (b) 150 μg/mL; (c) 200 μg/mL; (d) 300 μg/mL. HUVECs were seeded onto filters precoated with 40 μL matrigel on the upper surface in the presence or absence of λ-CO in transwell chambers. After a 24-h incubation, the cells invading the lower surface were fixed and stained and assessed under a microscope. (**B**) Quantification of inhibitory effects of λ-CO on HUVE cells invasion. The invaded cells were quantified by measuring the stained cells in three random areas per membrane. The experiments were performed three times. * indicates $P < 0.05$; ** indicates $P < 0.01$, compared to control.

In previous investigations, sulfated β-(1,4)-galactan saccharides including λ- and κ-carrageenan and their derivatives have been evaluated for their anti-angiogenic effect (*36*, *37*). They found that κ-carrageenan failed to inhibit experimental parameters relative to angiogenesis, but λ-carrageenan showed the anti-angiogenic ability. By our observation, κ-carrageenan oligosaccharides also expressed no effect on angiogenensis. These results indicated that, besides the molecular size, the degree of sulfation is also a critical structural parameter for the carrageenan oligosaccharides to inhibit angiogenesis. In the three types of carrageenan, the λ-type possesses the highest number of sulfonic acid groups, so it also showed the highest anti-angiogenic activity. This tendency is consistent with previous reports about other sulfate oligosaccharides (*18*, *19*). Previously, some general comments have been made about the structural requirements for sulfated oligosaccharides to inhibit angiogenesis. Clearly, oligosaccharide chain length is critical, with a high degree of sulfation and the nature of the backbone also being important. The oligosaccharide chain length and degree of sulfation were more important parameters than the sugar composition and type of linkage. Thus, diverse polyanionic molecules such as suramin (Nakajima et al., 1991) (*38*), polyanetholesulfonic acid, aurintricarboxylic acid, and other synthetic polycarboxylic aromatic compounds (Benezra et al., 1992) (*39*) all inhibited angiogenesis, despite their different backbone structure and chemical nature.

When comparing the effect of λ-CO with those of polysulfonated compounds as well as oversulfated and unmodified heparin, we found λ-CO inhibited angiogenesis in the CAM model in a dose-dependent manner; however, the concentration needed was higher than that of the other compounds tested. For example, suramin can exert an anti-angiogenic effect at 50 μg/egg with an inhibition of 46% (*7*), and LaPSvS1 can induce a strong anti-angiogenic effect at 50 μg/egg (*40*). However, in our experiment, we used 100−200 μg/egg of λ-CO to generate this effect. At 100 μg/egg, λ-CO showed an about 30% inhibitory ratio. So, λ-CO is a mild angiogenesis inhibitor.

It is evident that the activation and growth of endothelial cells is a prerequisite for vessel formation during angiogenesis. Therefore, the question arises whether the anti-angiogenic effect in the CAM model is correlated to the cytotoxic effect of λ-CO *in vitro*. Our data showed that at low concentration, at least lower than 125 μg/mL, there was almost no difference in cell growth and viability compared with untreated control (**Figure 4**). However, at high concentration (from 500 μg/mL to 1 mg/mL), λ-CO expressed different cytotoxic sensitivities toward different cell lines (**Figure 5**). In the incubation with HUVECs for 48 h, λ-CO (1 mg/mL) could inhibit the viability of cells about 60.23%, but it had only a marginal cytotoxic effect toward L-02. This property is important for this agent that may eventually be used therapeutically, because its differential killing of endothelial cells seldom affecting normal tissues is a highly desired beneficial feature for therapy. In addition, we observed from **Figure 5** that liver cancer cell Bel-7402 was slightly affected by λ-CO, which indicates that when a high concentration of λ-CO arrived at the tumor tissue, it may inhibit the proliferation of endothelial cells first and then attack the tumor

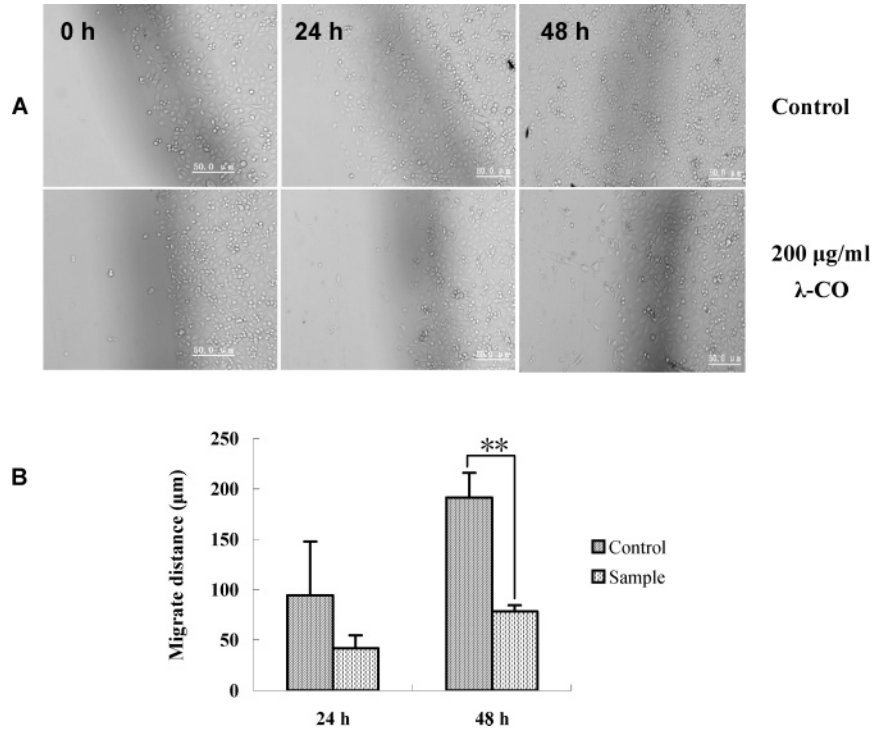

Figure 8. (**A**) Photographs showing the migration of HUVECs inhibited by λ-CO. (**B**) Quantification of migration distance of HUVECs. Migration of HUVECs was determined by wound assays. Confluent cell cultures were wounded. Subsequently, wound width was measured at certain time points. Means ± SD from three different experiments are shown. Asterisks represent statistical difference from control experiments performed in the absence of oligosaccharides ($P < 0.01$). The dark area is the scraping line.

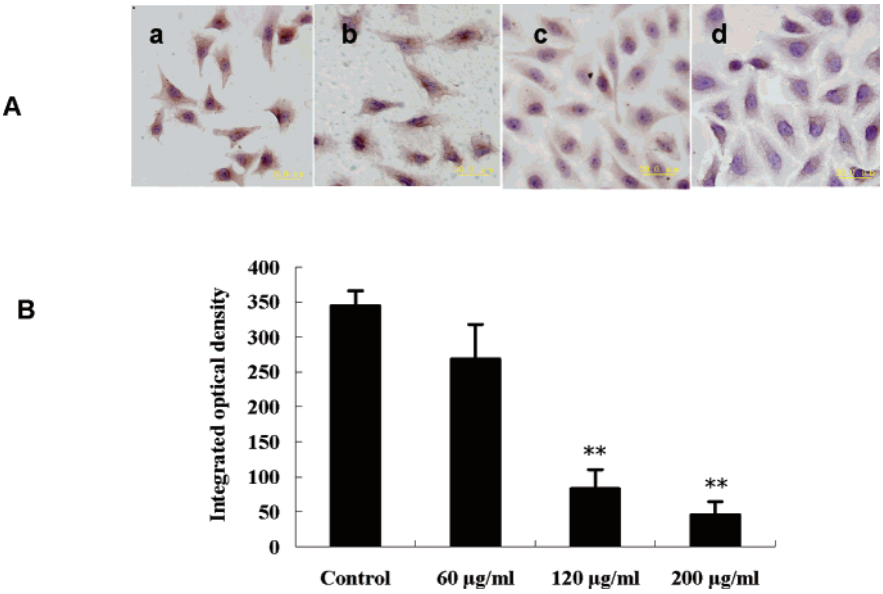

Figure 9. (**A**) Down-regulation of MMP-2 expression following exposure of HUVECs to λ-CO (24 h of culture): (a) control; (b) 60 μg/mL; (c) 120 μg/mL; (d) 200 μg/mL. (**B**) Quantification of inhibitory effects of λ-CO on MMP-2 expression. The expression level of MMP-2 in HUVECs was investigated using the techniques of immunohistochemistry and image analysis. The protein level was expressed as integrated optical density (IOD). * indicates $P < 0.05$; ** indicates $P < 0.01$, compared to control.

cells and normal cells. So, it may efficiently inhibit angiogenesis for the treatment of tumors.

Endothelial cells are the source of new blood vessels. In addition to their proliferation property, they also have a remarkable ability to invade, migrate, and differentiate. In this study we examine the role of λ-CO in regulating HUVECs' migration and invasion with a view to elucidating the potential action mode of these oligosaccharides involved in the angiogenesis process. We found that λ-CO possesses potent inhibitory activities not only on the invasion of HUVECs but also on their migration ability. In the wound assay, gradually fewer cells were present in the wound area when HUVECs were treated with λ-CO. These results assumed that the mechanism of λ-carrageenan oligosaccharides may be similar to that of most of the heparin mimetic compounds, including their undegraded form. These compounds have been reported to be used as potential antitumor and anti-inflammatory agents because they can block the degradation of heparin sulfate glycosaminoglycans (HSGAG) in the ECM and the cell surface by inhibiting the activity of heparanase (*41*).

However, besides the heparanase, MMPs also have been confirmed to have a significant role in endothelial cells' migration, invasion, proliferation, and attachment to one another (*42*). Among them, MMP-2 was reported to play a pivotal function in facilitating ECM remodeling and migration of vascular ECs (*43*, *44*). We therefore extended our investigations to examine the effect of oligosaccharides on the level of MMP-2 of HUVECs by immunocytochemical analysis to test whether λ-CO can exert its migration or invasion inhibitory effect by reducing the expression of MMP-2. As shown in **Figure 9**, the MMP-2 level was indeed altered by exposure to λ-CO in the HUVECs. These findings appear to rule out a significant role for attenuation of MMP-2 expression in λ-CO mediated inhibition of endothelial migration and invasion.

In conclusion, in this study, we have demonstrated, for the first time to our knowledge, that λ-carrageenan oligosaccharides could effectively inhibit angiogenesis in the CAM model and could block invasion and migration of human umbilical vein endothelial cells (HUVECs) by down-regulation of intracellular MMP-2 expression. The results suggest that antiangiogenesis of λ-carrageenan oligosaccharides may occur through the combined effects of inhibiting invasion, migration, and proliferation. Further studies on the heparanase inhibitory ability and the cytotoxic mechanism of λ-carrageenan oligosaccharides are underway. Moreover, studies relating to the pharmacokinetics and anti-angiogenic efficacy of these compounds in animals also have been initiated to determine their potential role as clinical drug candidates.

ABBREVIATIONS

LMW, low-molecular weight; λ-CO, λ-carrageenan oligosaccharides; HUVECs, human umbilical vein endothelial cells; CAM, chick chorioallantoic membrane.

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