Low Molecular Weight Chitosan Stimulation of Mitogenic Response to Platelet-derived Growth Factor in Vascular Smooth Muscle Cells

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Low molecular weight chitosan (LMWC) (a mixture of chitooligosaccharides with high degrees of polymerization; an average degree of polymerization is 6.8) stimulated mitogenic response to platelet-derived growth factor (PDGF) in a dose-dependent manner in cultured rat vascular smooth muscle cells, and a maximum effect was observed at 100 μg/ml. However, the mitogenic response was not induced when cells were incubated with LMWC alone. This stimulatory effect of LMWC on the mitogenic response to PDGF (a competence factor) appeared to resemble the effect of insulin as a progression factor. Chitooligosaccharides with higher degrees of polymerization were more effective, but α-glucosamine, chitobiase, and chitotriose were barely active. LMWC as well as PDGF induced protein tyrosine phosphorylation in vascular smooth muscle cells.

Chitin [(1→4)-linked 2-acetamido-2-deoxy-β-glucan] is widely distributed in nature, for example in crab and shrimp shells, insects, mushrooms, and the cell walls of yeasts and fungi. Chitosan is industrially produced by the N-deacetylation of chitin, and is also found in the cell walls of certain fungi. Chitosan and its derivatives have been evaluated for a number of medical activities and applications, and found to have anticoagulant, antiviral, antitumor, and adjuvant activities. It was also reported that interleukin-1 and colony-stimulating factor were induced when macrophages were stimulated with chitosan derivatives. Further, chitosan and its derivatives are shown to accelerate wound healing and to be useful for wound management. Chitosan is thought to be degraded in wound sites by the actions of lysozyme and other related enzymes (N-acetyl groups usually remain in part in chitosan, and lysozyme can attack chitosan by recognizing the remaining N-acetyl-d-glucosamine residues); however, details in function of chitosan and its hydrolyzing products (i.e., chitooligosaccharides) in wound sites have not been well explained.

Platelet-derived growth factor (PDGF) is one of the major mitogens in serum, and is responsible for proliferation of certain types of cells including fibroblasts and vascular smooth muscle cells (VSMC) in wound sites. PDGF occurs in three homo- and heterodimeric isoforms composed of two homologous polypeptide chains designated A and B, and these isoforms (AA, AB, and BB) exert their biological effects by binding to two types of cell surface receptors, α and β, with distinct binding properties. In VSMC, PDGF-AB and -BB act as potent mitogens but PDGF-AA is essentially inactive, because the PDGF-β receptor, which shows a high binding affinity only for the PDGF-B chain (but not for the A chain), alone is expressed in this cell type.

We have studied the biological effects of chitosan derivatives in cultured rat VSMC. In this paper, we report that water-soluble, low molecular weight chitosan (LMWC) stimulates mitogenic response to PDGF-BB in this cell type, although LMWC itself does not act as a mitogen. This stimulatory effect of LMWC may account, at least partly, for the acceleration of wound healing by chitosan.

Materials and Methods

Chemicals. LMWC (lactate salt; pentose, 34.3%; hexose, 18.4%; heptose, 16.4%; octose, 11.0%; nonose, 9.9%; decose, 6.7%; undecose, 3.3%), prepared by enzymatic digestion of chitosan, was a gift from Pas Corporation (Osaka, Japan). Human recombinant PDGF-BB homodimer and a monoclonal antibody to phosphotyrosine were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.), chitooligosaccharide chloride salts (bisiose, triose, tetraose, and hexaoe) from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan), and [methyl-3H]-thymidine (83 Ci/mmol) from Amersham.

Cell culture. Rat VSMC, kindly donated by Dr. Tadashi Inagami (Vanderbilt University, Nashville, TN, U.S.A.), were seeded in a 15-mm well plate (2 × 10⁵ cells/well) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. After reaching about 10⁶ cells/well, the cells were transferred to 1 ml of a 1:1 mixture of DMEM and Ham's F-12 medium containing 10 μg/ml transferrin, 10 ng/ml sodium selenite, and 1 μg/ml bovine serum albumin (BSA), and cultured for a further 2 days for serum deprivation. Then, the cells were stimulated with PDGF (25 ng/ml) in the presence of LMWC for 2 days, and cell growth (relative cell number) was measured with a water-soluble tetrazolium derivative (WST-1) by using a commercial kit (Dojin, Kumamoto, Japan). Absolute cell number was measured with a hemocytometer after cells were detached from a plate with trypsin.

Measurement of DNA synthesis. Cells in a 15-mm well plate (about 10⁶ cells/well), which had been cultured in 1 ml of the mixture of DMEM and Ham's F-12 medium for 2 days, were stimulated with PDGF in the presence of LMWC for 20 h. During the final 4 h [methyl-3H]-thymidine was added into the medium at 2 μCi/well, and radioactivity incorporated into newly synthesized DNA was measured by a published method.

Immuno blotting. Serum-deprived cells in a 90-mm dish (about 2 × 10⁶ cells/dish) were incubated with LMWC in 20 ml HEPES-NaOH buffer, pH 7.4, containing 130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose, and 1 mg/ml BSA, at 37°C for an appropriate time.
interval. After washing with ice-cold 20 mM HEPES-NaOH buffer, pH 7.4, containing 130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 2 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, and the cells were lysed with 20 mM HEPES-NaOH buffer, pH 7.4, containing 0.5% Nonidet P-40, 50 mM β-glycerophosphate, 0.1 mM orthovanadate, 10 µM molybdate, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml leupeptin, and 10 µg/ml aprotinin, at 4 °C. After centrifugation at 14,000 x g for 5 min, the supernatant was used as cell lysate. SDS-polyacrylamide gel (7.5%) electrophoresis was done by the method of Laemmli, and proteins in the gel were transferred to a nitrocellulose membrane by electroblootting. The membrane was treated with an anti-phosphoryrline monoclonal antibody (1 µg/ml) in 10 mM Tris-HCl buffer, pH 7.5, containing 5% BSA, 0.9% NaCl, and 0.1% Triton X-100, and tyrosine-phosphorylated proteins were detected by a chemiluminescent method using a commercial kit containing alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (Bio-Rad). Protein content in cell lysate was measured as described by Bradford using BSA as a standard.

Results and Discussion

Stimulation of PDGF-induced cell proliferation by LMWC

As has been reported previously, PDGF-BB acted as a potent mitogen in cultured rat VSMC, and cell number in a 15-mm well increased from 1.1 x 10^5 to 2.5 x 10^5 during exposure at 25 ng/ml for 2 days. When LMWC was present during the exposure, the PDGF-induced cell proliferation was stimulated by a dose-dependent mechanism (Fig. 1). The maximum effect of LMWC was observed at 100 µg/ml, and at this concentration cell number was increased 13.5-fold over control (PDGF alone). However, in the absence of PDGF LMWC did not show any effects even at a concentration as high as 200 µg/ml. To further clarify the effects of LMWC on the growth of VSMC, we examined DNA synthesis by measuring [methyl-³H]thymidine uptake. As shown in Fig. 2, DNA synthesis was induced by exposure to PDGF, and the level of the induction was substantially increased (about 1.7-fold) by the presence of LMWC at 100 µg/ml. However, DNA synthesis was not induced by LMWC alone.

The LMWC used in this experiment is a mixture of chitoooligosaccharides with high degrees of polymerization (DP) (composed of pentose, hexose, heptose, octose, nonose, decaose, and undecaose; average DP is 6.8). To discover the relationship between the size of chitoooligosaccharides and their function, stimulatory effects on PDGF-induced cell proliferation were examined with chitoooligosaccharides with lower DP. As shown in Fig. 3, chitotetraose and chitohexaose increased the cell number substantially (1.15-fold and 1.31-fold, respectively, over control (PDGF alone)), but the extents were lower than that observed with LMWC (1.41-fold in this experiment). In contrast, d-glucosamine, chitobiose, and chitotriose did not have significant effects. These results indicate that chitoooligosaccharides with higher DP are more effective in the range examined in this experiment.

The data presented here show that chitoooligosaccharides with high DP (including LMWC) increase mitogenic response to PDGF in cultured rat VSMC, though they do not act as mitogens. Nishimura et al. reported some water-soluble chitin derivatives (such as 6-O-carboxymethylchitin and 6-O-hydroxyethylchitin) as well as concanavalin A and lipopolysaccharide showed mitogenic activities in normal mouse spleen cells, but chitosan (a partially N-acetylated, water-soluble preparation) was inactive. However, they did not examine synergistic effects of chitosan on the proliferation of these cells induced by serum or other mitogens. Our data suggest that a receptor recognizing chitoooligosaccharides occurs on the surface of VSMC, and 4 or more d-glucosamine units are necessary.

![Fig. 1](image1.png)

**Fig. 1.** Dose-dependent Effect of LMWC on PDGF-induced Cell Proliferation.

Serum-deprived cells in a 15-mm well were incubated with various concentrations of LMWC in the presence (○) or absence (●) of PDGF-BB (25 ng/ml) for 2 days, and relative cell number was measured as described in Materials and Methods. Data (n=4, ±SE) are presented as a percentage of control (without LMWC or PDGF; absolute cell number counted with a hemocytometer was 1.1 x 10⁵ cells/well).

![Fig. 2](image2.png)

**Fig. 2.** Enhancement of PDGF-induced DNA synthesis by the Presence of LMWC.

Serum-deprived cells in a 15-mm well were incubated with LMWC (100 µg/ml) and/or PDGF (25 ng/ml), and the induction of DNA synthesis was measured by [methyl-³H]thymidine uptake as described in Materials and Methods. Data are presented as mean of four measurements (±SE).

![Fig. 3](image3.png)

**Fig. 3.** Stimulation of PDGF-induced Proliferation by Chitoooligosaccharides with Different Size.

Cell proliferation was induced by PDGF (25 ng/ml) in the presence of chitoooligosaccharides with different DP (each 100 µg/ml), and relative cell number was measured after 2 days as described in Materials and Methods. Data (n=4, ±SE) are presented as a percentage of control (without chitoooligosaccharides, plotted at the position of DP of "0" for convenience).
for recognition; however, details still remain to be studied.

**Mechanism of the stimulatory effect of LMWC**

PDGF is regarded as a competence factor that needs progression factors to cause a complete cell proliferation. Indeed, mitogenic response to PDGF is markedly increased by the presence of insulin, a progression factor, in VSMC. When cell proliferation was induced by PDGF in the presence of insulin, a synergistic effect of LMWC was not observed (Fig. 4). Further, cell proliferation was not induced in the absence of PDGF even if both insulin and LMWC were added simultaneously. Thus, LMWC appears to mimic insulin as a progression factor in VSMC.

A tyrosine kinase domain occurs in both types (α and β) of the PDGF receptors. The intrinsic tyrosine kinase is activated in response to ligand binding, and the activation is recognized to be the first and essential event for the mitogenic signal transduction of PDGF. When VSMC were exposed to LMWC, as shown in Fig. 5, a number of tyrosine-phosphorylated proteins appeared in 5 min, and after 10 min the phosphorylation reached a maximum level. The extent of tyrosine phosphorylation induced by LMWC was comparable to that observed in cells elicited by PDGF, although the PDGF-β receptor (180 kDa), which was autophosphorylated in response to ligand binding, was barely phosphorylated by the action of LMWC. These results indicate that tyrosine kinases are activated by stimulation with chitoooligosaccharides in VSMC, and we submit a hypothesis that the activation of tyrosine kinases is an important step in signal transduction by chitoooligosaccharides. This notion is supported by evidence that receptors for some progression factors including insulin are involved in the superfamily of tyrosine kinases; however, it is unclear whether a receptor for chitoooligosaccharides has an intrinsic tyrosine kinase activity.

It has been reported that chitosan, when attacked on wound sites, accelerates wound healing. Chitosan on wound sites may be hydrolyzed to form chitoooligosaccharides by the actions of lysozyme and other related enzymes found in serum and tissues. We observed an increase of serum lysozyme activity upon injecting a mixture of chitoooligosaccharides intravenously in rabbits, suggesting that the serum lysozyme level increases around wound sites attached with chitosan in response to an increase in the level of its products. PDGF, which is produced by platelets (the AB isoform in human platelets but the BB isoform in procine ones), is responsible for proliferation of VSMC and fibroblasts in wound sites. Thus, the stimulatory effect of chitoooligosaccharides on the mitogenic action of PDGF presented in this paper may be attributed, at least in part, to the acceleration of wound healing by chitosan.

**References**


