Study on the mechanisms of wound healing acceleration by chitin and chitosan

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Chitin, a poly-β (1→4)-*N*-acetyl-D-glucosamine, is well known as a cell wall component of fungi and as a skeletal material of invertebrates. Chitosan is prepared by chemical *N*-deacetylation of chitin. Chitin and chitosan have been observed to accelerate wound healing properties and the attainment of a good-looking healing skin surface. However, the precise mechanisms of these actions are unknown.

The scope of the present work was to determine fibroblasts, endothelial cells and macrophages responses to chitin and its derivatives in order to clarify the effect of chitin and chitosan on wound healing.

Chitin and its derivatives showed almost no acceleratory effect on the proliferation of cultured fibrobrasts. On the contrary, high-concentration (500 mg/ml) D-glucosamine cultures supplemented with or without 10% fetal bovine serum (FBS) showed a significant ($p<0.05$) reduction in the rate of proliferation of L929 fibroblast cells relative to control. High-concentration chitosan cultures supplemented with 10% FBS showed a significant ($p<0.05$) reduction in the rate of L929 fibroblasts proliferation. However, the inhibition of cell proliferation by high concentrations of chitosan did not show in the cultures without FBS. Interluekin (IL)-8 was induced in the supernatants of rat primary cultured dermal fibroblasts stimulated with chitin and its derivatives. Chitin and its derivatives did not stimulate the production of IL-6 by mouse dermal primary cultured fibroblasts. IL-1α, IL-1β and tumor necrosis factor (TNF)-α were not detected in the fibroblast supernatants.

Chitin and its derivatives had no effect on the proliferation of cultured human umbilical endothelial cells (HUVEC). IL-8 was induced in the supernatants of HUVEC stimulated with chitin and its derivatives. Chitosan stimulated the production of IL-6 and TNF-α from HUVEC. Compared to chitosan the other materials tested in the present study showed less effect in the stimulation of IL-6 and TNF-α production.

In order to analyse the detailed mechanisms of the activation ability on macrophages, residental peritoneal macrophages were prepared and stimulated with several polysaccharides such as chitin, chitosan, and their derivatives. It was demonstrated that (i) chitosan and its derivatives treatment induced apoptosis on peritoneal macrophages, (ii) expression of activation markers, such as major histocompatibility complex (MHC) class I, class II and Fc receptors, transferrin receptor, mannose receptor, Fas, and macrophage inflammatory protein (MIP)-2 were induced after chitosan treatment but expression of MHC class I and II molecules alone were induced by chitin and low-molecular soluble chitosan, and (iii) additionally, apoptosis induced by chitosan was mediated by the Fas signaling pathway.

These observations support the assumption that fibroblast and endothelial cell proliferation are accelerated indirectly by chitin and its derivatives when these materials are used *in vivo*. *In vivo* findings of angiogenesis and migration of neutrophils may be due to persistent release of IL-8 from fibroblasts and endothelial cells. Chi-

tosan is an activator of macrophages and it is therefore suggested that chitosan, through MIP-2 production, MHC class II-$CD4^+$ lymphocyte interactions, and Fc-receptor-mediated and mannose receptor-mediated phagocytosis, may accelerate wound healing.

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Studies on the establishment of micrometastasis models using bacterial *lacZ* gene-tagged tumor cells and chemosensitivity of micrometastasis to an anti-cancer agent

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lacZ gene encodes Escherichia coli β-galactosidase, whose enzymatic activity can be simply detected by staining with the chromogenic substrate 5-bromo-4-chloro-3-indoyl-β-galactopyranoside (X-Gal), has been recently reported to greatly facilitate detection of metastasis in their earliest stages of development. In the present study, the author transfected rat deffe-rentiated adenocarcinoma cells and mouse lung carcinoma cells with a *lacZ* gene to establish micrometastasis model, and sequential events of micrometastasis formation by these cells were investigated. In addition, the therapeutic effect of anti-cancer drug UFT on micrometastasis was examined using mouse lung carcinoma cells transfected with the *lacZ* gene.

In chapter 1, a micrometastasis model was established using a rat differentiated prostatic adenocarcinoma, designated PLS301Z, transfected with the *lacZ* gene. The morphology, tumorigenicity and metastatic ability of PLS301Z were comparable to those of the parental cells. Micrometastatic foci could be specifically detected at the single cell level after X-Gal staining with a dissecting microscope. After intravenous injection, the number of X-Gal positive foci in lung decreased progressively to a steady state level (less than 1% of injected cells) by 4–7 days, while the size of persisting positive foci started to increase from 4 days after inoculation as demonstrated by image analysis. X-Gal and BrdU double staining revealed that BrdU labeling indices of X-Gal positive cells decreased transiently at the 2 day time point and increased again from 4 days after inoculation. Type IV collagen immunostaining showed the tumor cells to be surrounded by a basement membrane intravascularly at the time point when they started new growth. Electron microscopy confirmed that, 2 days post injection, most tumor cells were degenerative or dead, but on day 4, persisting tumor cells formed multicellular clumps in contact with the vascular basement membrane inside vessels. These results indicate that PLS301Z cells begin to grow intravascularly depending upon the presence of a basement membrane before extravasation at the initial stage of micrometastasis formation.

In chapter 2, sequential events in micrometastasis formation including entry into the blood circulation and arrest, extravasation and initial growth in the lung was investigated using bacterial *lacZ* gene-tagged Lewis lung carcinoma