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Chitinase Induces Lysis of MCF-7 Cells in Culture and of Human Breast Cancer Xenograft B11-2 in SCID Mice

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Abstract. *Background: It has long been known that the polycarbohydrates on the neoplastic cell surface are different from those on normal cells; differences which allow one to attack tumor cells selectively. Although the exact differences between tumor cells and normal cells are still not clearly known, research into these differences is ongoing for anticancer drug development. Materials and Methods: The human breast cancer cell line MCF-7 in culture and human breast xenograft* B_{11}*-2 in SCID mice were used in our observations. Two different samples of chitinase from different bacteria were tested in the experiments. Optical observation of regular H & E-stained tumor tissue sections and observations by transmission electron microscopic techniques were used in this study. Results: MCF-7 breast cancer cells in culture showed structural damage within 7 hours after 1.3 unit/ml of chitinase was added to the medium, while normal mice spleen cells did not. The transplanted* B_{11}*-2 xenograft tissue in mice started to lyse 12 hours after chitinase was injected; the size of the tumor gradually reduced and finally a scab was formed, which came off the skin a few days later. All the tested tumor-bearing mice survived and these cured mice had no tumor re-growth during the following 1-year observation period. Conclusion: Chitinase selectively lysed the tumor cells in vitro and in vivo. Injected chitinase destroyed the tumor tissue and cured the mice. The further development of this type of treatment and of the mechanisms of chitinase action are discussed.*

Tumorigenesis is accompanied by marked changes in the pattern of gene expression and post-translational modifications. These changes often lead to changes in cellular phenotype and membrane composition. It is well known that the polycarbohydrate structures and their organization on the surface of the neoplastic cells frequently vary during tumor cell growth and differentiation (1-5). Essentially, all tumor cells display carbohydrate profiles distinct from those of non-transformed cells from the same tissue (6-11). One of the common changes in cell surface carbohydrates in tumor cells is the appearance of high molecular weight glycoproteins that are not found in normal cells (12, 13). The changes in carbohydrate composition are even more pronounced in tissues of metastatic lesions than in primary tumors (14, 15). Evidence shows that the oligosaccharides of these glycoproteins play an important role in determining the biological behavior of the tumor such as, for example, metastatic potential (16-18). The change also seems to be related to cell signal transduction, cell communication and membrane function (19-23). Such changes in the tumor cell membrane can be recognized by the immune system and distinctive carbohydrates have been identified as tumor-associated antigens (24). Tumor-specific glycoproteins and glycolipids have been evaluated in clinical trials as potential antigens for use in vaccines for tumor immunotherapy (25, 26). The differences in surface carbohydrate composition between the tumor cells and the normal cells, therefore, present a good opportunity for a new strategy of searching for low toxicity tumor treatment and selective tumor cell attack (5, 27-31). However, although much effort has been devoted to the study of selective attacks, the results to date have been, at best, unsatisfactory.

Chitinase is a carbohydrate hydrolase. Its natural substrate is chitin, which consists of a group of polycarbohydrates rich in N-acetyl-D-glucosamines. Chitin is the primary component of the exoskeleton in a large number of organisms, including the cell walls of fungi and of some algae and the shells or cuticle of arthropods. Normal mammalian cells contain no chitin, nor has it been reported in cancer cells.

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Interestingly, when chitinase was added into the culture medium of cultured cancer cells, a significant cell surface damage was observed, ultimately leading to cell death. As a result of this observation, we have undertaken further evaluation of the antitumor efficacy of chitinase in a number of animal tumor models. In this paper, the effects of chitinase on the human breast cancer cell MCF-7 in culture and human breast cancer xenograft B_{11}-2 in SCID mice are reported.

Materials and Methods

Reagents. The culture medium D-MEM/F-12 with 15 mM HEPES buffer, L-glutamine and pyridoxine hydrochloride were obtained from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum was purchased from Gemini Bio-Products, Inc. All the other materials, unless otherwise stated, were from Sigma (St. Louis, MO USA). Two chitinase samples, prepared from different microbes, were tested in our study. One was a lyophilized powder, extracted from *Serratia marcescens*; the other was also a lyophilized powder, extracted from *Streptomyces griseus*.

MCF-7 cells in culture. The human breast cancer cell line MCF-7 was cultured in D-MEM/F-12 medium added with 10% preheated fetal bovine serum and a final concentration of 50 units/ml penicillin G and 50 μg/ml streptomycin. The chitinase in pH 7.4 PBS sample solution was directly added into the culture medium in the cell culture wells with serum in the medium. For preparation of the cell sample for electron-microscopic observation, the cells were planted onto small round microscope cover glasses (one cm in diameter). A 24-well cell culture plate was used in the experiment; for each well $2x10^5$ cells in 2 ml culture medium were planted, and then the plate was kept in a 37˚C, 5% CO_2 air-phased incubator for two days, at which time the cells had become firmly attached on the cover glass surface. The cell confluence was about 80%. After changing the medium, the chitinase in PBS solution was added and the plate was shaken thoroughly to ensure that the enzyme was completely dispersed in the medium. For H & E staining, the cells on the cover glass were fixed with acetone and stained following the routine procedures. The fixing and sample section procedures for electron microscopic observation are described in the paragraph describing the xenograft.

Human breast cancer xenograft B_{11}-2 in SCID mice. The method for developing the primary human cancer xenograft model in SCID mice and the results of the mark antigen tests will be reported in detail in another paper. Basically, the procedure of transplantation was as follows: human breast cancer tissue was obtained from surgery, and immediately dipped in RPMI-1640 culture medium without serum. After it was cut into 1-mm^3 pieces, and then transplanted into SCID mice by a surgical operation behind the fat pad, or subcutanously. The success rate of implantation of the breast cancer tissue was not high, at only about 20%. The B_{11}-2 xenograft tissue line used in this observation was a ductal carcinoma and had been transplanted in SCID mice twice. The first time was from human patient to mouse and the second time was from one mouse to a group of next generation mice. It has been proven in our laboratory that the immunological characteristics of these human cancer xenograft tissues in SCID mice had not changed, or at least they were very similar to those of the original tissue sample. The tissue structure was also similar to the original tissue, except that the cells which constituted the blood vessels in those xenograft tissues were from the host mice.

Chitinase solution in pH 7.4 PBS was freshly prepared before use, and directly injected into the center of the cancer xenograft tissue. At special time-points, the mice were sacrificed with CO_2 gas and the cancer xenograft tissues were take out immediately and cut into four parts with two vertical cuts through the center of the cancer tissue. Parts 1 and 3 of the tissue were fixed in buffered formalin solution, and processed routinely for paraffin-embedding, hematoxylin and eosin staining and used for morphological evaluation. The parts 2 and 4 of the xenograft tissue were fixed in freshly made 1% glutaraldehyde in 0.01 M cacodylate buffer overnight at 4˚C. On the second day, the tissue samples were further fixed by 1% OsO_4 at 4˚C for 60 minutes. The samples were washed three times with cacodylate buffer, and then dehydrated in 60, 70, 80, 95 and 100% ethanol. The dehydrated tissue samples were washed twice with propylene oxide and immersed in po/eponate (1:1 mixture) overnight at room temperature. The po/eponate mixture was replaced with 100% eponate, and the sample polymerized at 70˚C. The polymerized samples were sectioned at 50 nm with a diamond knife, mounted on cupric grids and stained for 10 minutes with an aqueous 5% uranylacetate, followed by one minute with Sato's lead stain. The samples were examined under transmission electron microscopy with a Philips CM 10 microscope.

For electron microscopic observation, the MCF-7 cell samples on the cover glasses obtained from culture were fixed with freshly made 1% glutaraldehyde in 0.01 M cacodylate buffer overnight, then the cells in the buffer were scraped off from the cover glass sheet with a plastic scraper. The cells were then collected by short time low speed centrifugation in a microcentrifuge tube, and then processed by the same procedures as the xenograft tissue samples.

Results

Chitinase induced MCF-7 cell lysis. A final concentration of 1 unit/ml chitinase was enough to induce obvious lysis of MCF-7 cells in 12 hours. Figure 1B shows the cell structure change in a MCF-7 cell cultured for 7 hours in culture medium which contained 1.3 units/ml chitinase by transmission electron microscope. Comparing B to the control cell A in Figure 1, which was cultured under the

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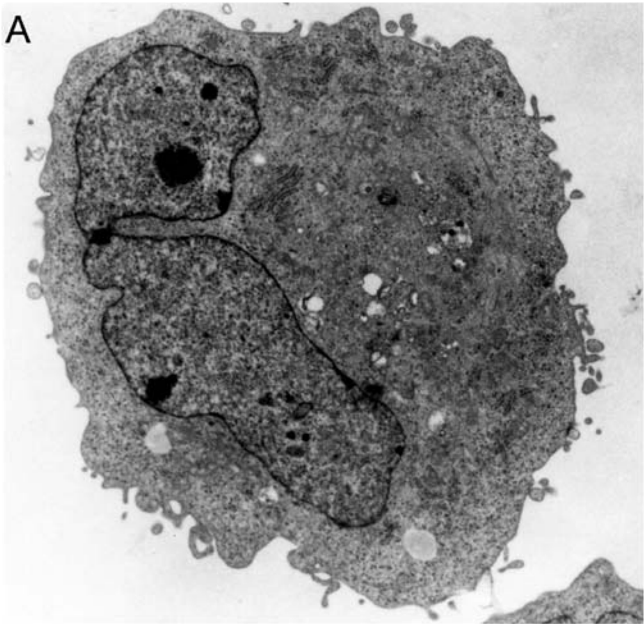
Figure 1. *A and B are transmission electron microscopic pictures of human breast cancer MCF-7 cell in culture. A, No chitinase treatment control. B. Cells were cultured in chitinase 1.3 units/ml added to the medium for 7 hours. C and D are human breast cancer B_{11}-2 xenograft tissue sections with H & E staining. C. Control, with 0.2 ml PBS solution injection. D. Sample was taken at 24 hours after 5 mg/0.2 ml chitinase solution injection. E and F are the same B_{11}-2 xenograft tissue, observed by transmission electron microscope. E. Control with PBS injection. F. Tissue sample was taken at 24 hours after chitinase injection.*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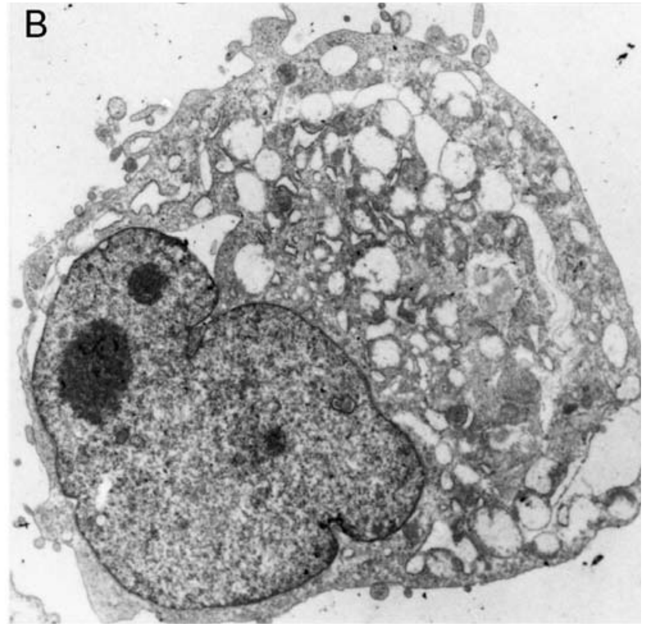
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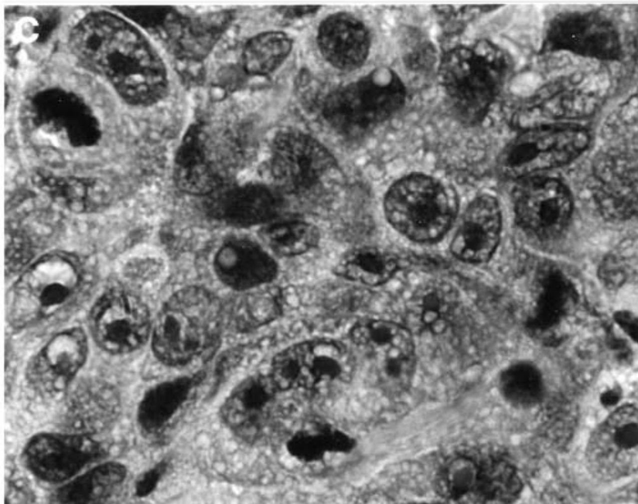
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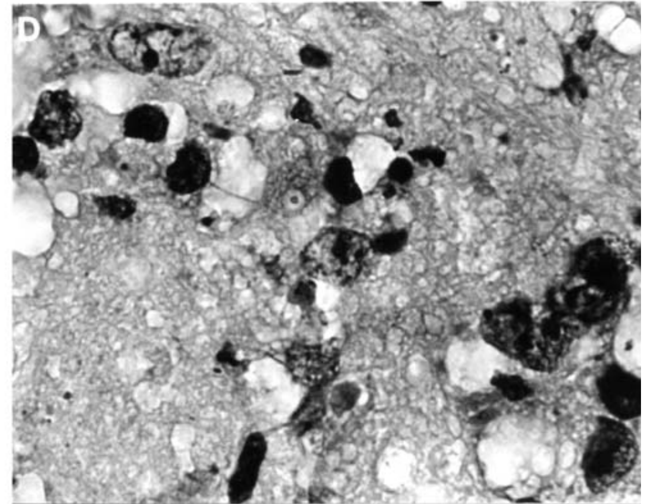
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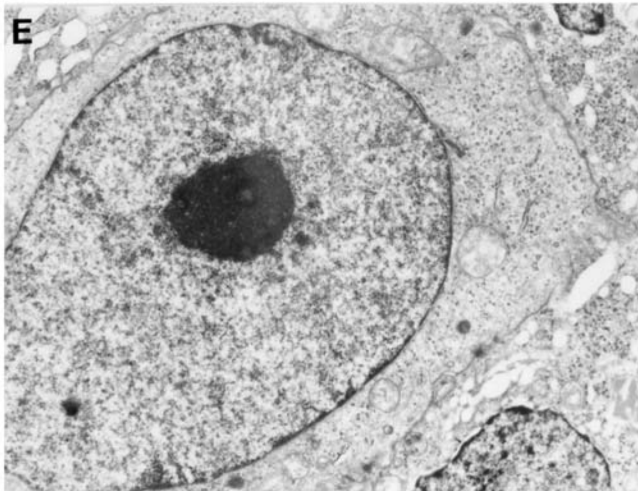
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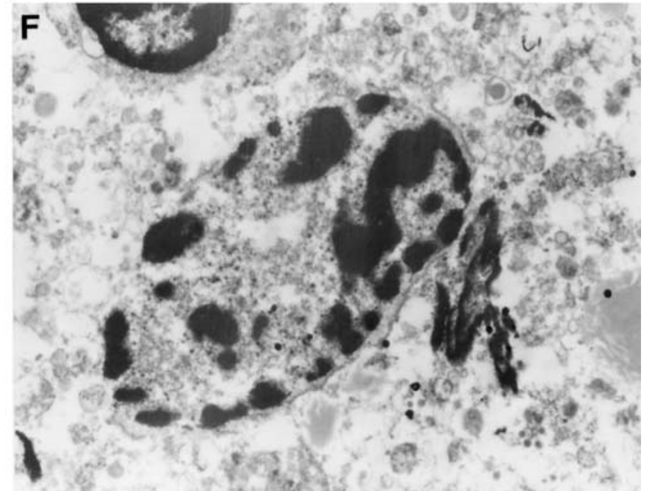
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same conditions but without chitinase; the main damage is seen in the cytoplasm and the cell surface membrane. After chitinase treatment, the cell cytoplasm was full of various sized vacuoles and most of the cell organelles were no longer visible. However, the nuclei of the cells showed no significant changes. Lipid granules were scattered in various places.

Normal mouse spleen cells were used as normal cell controls, under the same conditions and treatment. After H & E staining, the spleen cells showed no changes for three days and the cell numbers did not decrease.

Chitinase induced necrosis in human breast cancer xenograft B_{11}-2 in SCID mice. The B_{11}-2 breast cancer xenograft was allowed to grow to a size about 0.5 cm in diameter, then 5 units of chitinase in 0.1 ml pH 7.4 PBS solution was injected into the tumor. A few hours later, the color of the tumor turned dark and the tumor size contracted later. A couple of days later, the tumor finally became no more than a scab left on the skin. The scab came off the skin about 10 days later. Some large xenograft tumors required a second injection to be destroyed.

Figure 1C shows the control B_{11}-2 xenograft tissue after H & E staining and D shows the damaged B_{11}-2 tissue, that sample having been taken at 24 hours after chitinase injection. Figure 1C shows the breast cancer structure without treatment with some dividing cancer cells. After chitinase treatment (Figure 1D), the structure of the tumor is damaged, the cells are broken, and only condensed nuclei are left. Figure 1E shows the control untreated B_{11}-2 xenograft tissue observed under the transmission electron microscope. The cell nucleus is clearly visible, as well as the structure between the neighbour cells. By 24 hours after chitinase injection, the B_{11}-2 xenograft tissue was severely damaged, the cell membranes were broken and the cell cytoplasm had leaked out. The nuclei of the cells were also damaged.

During the treatment, the mice showed no symptoms of toxification. They slept, ate and played actively. The cured mice had a scar on their skins, at the location where the tumor had been, with little or no hair on the scar site for a while.

Discussion

Interestingly, in cancer studies, a number of researchers have reported that sometimes acute bacterial infections in the host could induce various types of cancer to undergo "spontaneous" regression (32-36). In these cases, multiple causes may be involved: the bacteria may produce toxins which may inhibit the cancer cells; the fever caused by infection may not be good for the cancer cells, or the bacterial infections induced the patients to develop immunity which cross reacted to the cancer cells *etc.* Certainly, during bacterial infection, the macrophage cells of the patient will be stimulated and some of them will produce leukocytes and a number of toxins against the invasion. It has been reported that chitinase is commonly produced by cells at the time of bacterial, fungial and nematodal intervention (37, 38). Here, we have seen the effect of chitinase on the cancer cells, and we propose that the chitinase is possibly also involved in those anticancer reactions.

People have tried to use carbohydrate from cancer cells to stimulate the host animals' defensive system against cancer (25-26). They have also used polysaccharides from bacteria or even plants to inhibit the growth of transplanted tumors in mice (39-41). Chitin, chitosan or short oligosaccharides have been used most often for this purpose. It has been proven that in N-acetylchito-oligosaccharides, the polymer size from the tetra-N-acetrylchitotetraose to hepta-N-acetylchitoheptaose display the most strong anticancer effect in mice, and that hexa-N-acetylchitohexaose and chitohexaose are the best compounds for stimulating the mice to act against the growth of transplanted tumor cells. Injection 5 times of 100 mg/kg of these substances can protect 100% of mice from sarcoma 180 solid tumor transplantation (42, 43). Interestingly, it has been observed that those oligosaccharides actually have no direct effect on the tumor cells. It has been found that injection of hexa-N-acetylchitohexaose can protect normal mice from *Candida albicans* infection. However, tumor-bearing mice already exhibited the same level of protection against *Candida albicans*, and did not need hexa-N-acetylchitohexaose. It has also been reported, that treating melon seedlings with chitin oligosaccharides elicited chitinase activity (44), but so far similar observations have been made in normal animals.

In our observations, two different chitinase samples, extracted and purified from different bacteria, were compared. The results were almost the same. In a previous abstract (45), we reported that not only breast cancer is affected by chitinase, but also human lung cancer, colon cancer, bladder cancer, melanoma and sarcoma cancer xenografts are all sensitive to chitinase treatment. Only the human prostate cancer line we developed, which grew slowly in SCID mice, showed a lower response to chitinase treatment. We suggest that new polycarbohydrates which react with chitinase are present on the surface of many cancer cells. After the carbohydrate parts of these glycoproteins or glycolipids are cross digested by chitinase, their original function is damaged, and the tumor cells die. As normal mammalian cells do not contain chitin, theoretically chitinase treatment will not hurt them.

The real mechanism of the anticancer effect of chitinase is not clear yet, thus future studies are necessary.

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