Chinese Journal of Cancer

Review

The mechanism of the anticancer function of M1 macrophages and their use in the clinic

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Abstract

M1-type macrophages are capable of inducing lysis in various types of cancer cells, but the mechanism of action is unclear. It has been noted that an "unknown protein" produced together with protease by activated macrophages is responsible for this action. Activated M1 macrophages have been recently reported to produce family 18 chitinases, all of which have been named chitotriosidase. Our experiments have demonstrated that family 18 chitinases work together with proteases and can damage various cancer cells both *in vitro* and *in vivo*. Thus, in this article, we suggest that the 50-kDa chitotriosidase is the reported "unknown protein". In addition, we discuss how to properly stimulate activated M1 macrophages to produce 50-kDa chitotriosidases and proteases for destroying cancer cells. Because family 19 chitinase has recently been reported to kill cancer cells, we also discuss the possibility of directly using human family 18 chitotriosidase and the humanized plant family 19 chitinase for cancer treatment.

Key words Chitotriosidase, protease, macrophages, anticancer

Peripheral blood macrophages play a pivotal role in the anticancer function of the immune system[1-3], but the mechanism by which activated macrophages kill cancer cells is unclear. Many people believe that nitric oxide (NO) or hydrogen peroxide (H_2O_2) are responsible for killing cancer cells because these compounds are produced by activated macrophages[4]. However, this explanation is not supported by solid evidence. Scientists have previously indicated that an "unknown protein" is produced by activated macrophages and works together with proteases to induce damage to the cancer cell surface[5,6], but this protein has not yet been identified. Failure to identify the nature of this unknown protein has restricted the use of macrophages in cancer therapy.

In our study on the differences between the surfaces of cancer cells and normal cells, we found that 0.5 unit/mL of family 18 chitinases (Sigma C7809 or C6137) can induce surface lysis of various types of cultured cancer cells. As a result of chitinase-induced lysis, cancer cells died within 24 h whereas normal cells were not killed. In addition, a single intratumoral injection of 5 units of family 18 chitinases killed a variety of human cancers in SCID mice when the tumor xenografts were approximately 0.3–0.5 cm^3 in size. When the tumor xenografts were larger, more than one injection was needed to kill all tumor cells. After the injection, the cancer tissue turned dark, and the tumor xenograft contracted for a few days. The tumor then became a black crust, which came off approximately 10 days later. Tumor-free mice were observed for 1 year, and cancer did not recur during this period[7,8]. Prior to a 2007 study conducted by Sanders *et al.*[9], it was unclear whether family 18 chitinases alone could kill cancer cells. Their study on the mucolytic activity of bacterial and human chitinases found that recombinant family 18 chitinase alone had no or only low mucolytic activity. However, when the recombinant family 18 chitinase was mixed with a low concentration of protease, the lytic activity increased markedly, although the protease alone had only slight mucolytic activity at this low concentration. Sanders *et al.*[9] also showed that the family 18 chitinase provided by Sigma (which we also used in our anticancer tests) contains protease and that the high mucolytic activity of family 18 chitinases was due to protease contamination. We now believe that the anticancer action of the bacterial family 18 chitinases produced by Sigma

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doi: 10.5732/cjc.012.10046

are actually the result of the combined action of family 18 chitinases and proteases[10].

It has been reported that the activated peripheral blood macrophages of humans and some other mammals are capable of synthesizing and secreting three family 18 chitinases, with molecular weights of 50 kDa, 39 kDa, and 40 kDa, which have been named 50-kDa, 39-kDa, and 40-kDa chitotriosidase, respectively[11,12]. These chitotriosides are components of the innate immune response and act against bacterial infection[13,14]. The 50-kDa chitotriosidase contains a chitin digestive center at the N-terminus, an 11-kDa chitin-binding domain at the C-terminus, and a linker structure between the N and C termini. After being synthesized, part of this 50-kDa chitotriosidase is truncated near the linker by protease to form the 39-kDa chitotriosidase and an 11-kDa chitin-binding peptide [15]. There is a specific mRNA in activated macrophages that generates the 40-kDa chitotriosidase, but its structure is similar to that of the 39-kDa chitotriosidase[12]. Because the 39-kDa and the 40-kDa chitotriosidases do not have a chitin-binding domain, they cannot react to the native form of chitin and, therefore, cannot kill fungal cells[16,17]. At the present time, synthesized water-soluble chitin-like compounds and chemically treated natural chitin are used as substrates for the chitinase activity assay. These substrates can be digested directly by the chitin digestive center of the chitotriosidase and do not need the chitin-binding domain to be involved. Thus, this type of assay is not able to distinguish the 39-kDa or 40-kDa chitotriosidase from the 50-kDa chitotriosidase. The inability to distinguish these chitotriosidases has caused confusion and has led to a misunderstanding of macrophage function.

It has been reported that the structures of family 18 chitinases are remarkably homologous in bacteria, humans, plants, and even in parasites, such as nematodes[18]. Based on these facts, it is now reasonable to suggest that the 50-kDa chitotriosidase may be the reported "unknown protein"[5,6].

Because it has been reported that a number of serine proteases, such as elastase, collagenase and plasminogen activator, are synthesized and secreted by activated macrophages, similar to chitotriosidases[5,6,19-21], and that "cell-to-cell contact" is the manner in which activated macrophages attack cancer cells[20-22], at the time activated macrophages attack cancer cells, the concentrations of chitotriosidase and protease on cell surface will be high enough to induce cancer cell lysis.

Based on these discussions, we believe that it may be possible to use human macrophages to kill cancer cells if we properly stimulate activated M1 macrophages.

A number of stimulation methods have been reported, including the use of bacterial lipopolysaccharide (LPS)[23-25], β-glucan[26], glycolipids of Mycobacterium bovis Bacillus Calmette-Guerin(BCG)[27], bacteria DNA-based vaccination[28], and chitin derivatives[10]. However, the results have not been fully satisfactory thus far. In this paper, we suggest suitable ways to stimulate activated M1 macrophages to kill cancer cells.

The Type of Macrophage Activation Changes During Cancer Development

Lines of evidence have indicated that there are two types of immunologic responses in the human body and in animal models: T helper 1 (Th1)-based and T helper 2 (Th2)-based responses. In the Th1 response, macrophages undergo M1-type activation, synthesizing chitotriosidases, protease, NO, H_2O_2, and other chemicals used to kill invaders, such as fungi, viruses, and bacteria. Cancer cells have also been shown to be destroyed by the Th1 response. In the Th1 mediated anti-cancer response, interferon-γ (INF-γ), interleukin-12 (IL-12), IL-2, and tumor necrosis factor-α (TNF-α) play major roles[29,30]. However, the over-activation of Th1 immunity sometimes causes certain types of autoimmune problems[31]. In the Th2 response, IL-4 and IL-10 play major roles, and macrophages undergo M2-type activation[29,30]. The mechanisms that control the immune response are not yet known; however, dendritic cells[32], $CD4^+CD25^+$ regulatory T cells[33-35], and T helper 17 cells[36] are involved.

During the early stages of cancer growth, some products from cancer cells and the tissue damage caused by invading cancer cells stimulate the Th1 immune response[37,38]. Because cancer cells are, on the whole, very similar to normal cells, $CD4^+CD25^+$ regulatory T cells, and possibly other types of regulatory cells[39], start to develop and prevent the over-activation of the Th1 response. These regulatory cells secrete IL-4, IL-10, IL-13, and so on, to change the immune response from a Th1 to a Th2 response[40,41]. Under the Th2 response, the macrophages are of the M2-type[42-44] and no longer produce chitotriosidases. The job of M2 macrophages is to remove dead cells, remodel damaged tissue, and stimulate new blood vessel growth. These actions actually help the cancer tissue to grow[45-48].

As the number of cancer cells increases, the T regulatory system gets stronger quickly, and T regulatory cells not only occupy the tumor tissue but also spread into the neighboring lymph nodes and spleen[41].

How to Properly Stimulate M1 Macrophages in Cancer-bearing Animals and Patients

Because only activated M1 macrophages produce

50-kDa chitotriosidases, we need to convert the Th2 response to the Th1 response in patients with cancer. To successfully induce the Th1 response, three points need to be followed. First, Bretscher *et al.*[49,50] have noted that the amount of the stimulator used to stimulate the Th1 response must be appropriately low. In their study of changing Leishmania-susceptible BALB/c mice to Leishmania-resistant mice, they showed that the amount of parasites used for the first inoculation must be less than 3.3×10^3 promastigotes per mouse for the mice to build up a Th1 response. BALB/c mice injected with fewer than 3.3×10^3 promastigotes not only survived the inoculation but also became resistant to the next high-dose Leishmania inoculation. However, if the first inoculation used more than 3.3×10^4 promastigotes, the mice developed a typical Th2 response, started to produce IgG1 antibody and were still susceptible to the next inoculation.

Hosken *et al.*[51] further proved in culture that in the presence of dendritic cells or activated B cells, the concentration of antigen directly determined whether naïve $CD4^+$ T cells would develop the Th1 or Th2 response. Only within a special range of concentrations of the antigen would naïve T cells develop the Th1 response and produce a large amount of IFN-γ and little IL-4. When the antigen concentration was higher than that range, or sometimes when it was too low, naïve T cells would develop the Th2 response and produce a large amount of IL-4 and little IFN-γ.

A similar result occurred during the treatment of sarcoma-180-transplanted ICR mice[52]. When the mice were given subcutaneous injections of a mushroom component called lentinan with a dose of 1 mg/kg·day for 10 days, their tumors completely regressed. However, when the lentinan dose was 80 mg/(kg·day) for 5 days, no tumor regression occurred[52].

The second point is that even when using the right amount of stimulator, the total number of stimulations is also important.

Virulizin is a product extracted from bovine bile that has been found to possess antitumor activity in a variety of human tumor xenografts in mice[53,54]. When a suitably low dose of virulizin was injected intraperitoneally every day for 3 to 5 days, the serum levels of IL-12α and IL-12β increased by 2.3- and 2.6-fold, respectively. The infiltration of macrophages and natural killer cells increased in the tumor tissue, and the tumor size decreased. When virulizin was given together with LPS in the culture medium *in vitro*, activated macrophages produced more TNF-α[55].

However, when the mice were given continuous injections of the same dose of virulizin for 4 weeks, a typical Th2 response developed: the expression of IL-17 increased, a significant number of eosinophils were recruited to the tumor tissue, and the anticancer effectiveness decreased[56].

The third point is that at the time of Th1 response stimulation, consuming large amounts of fruits, vegetables, and concentrated tea should be avoided. It has been confirmed recently that compounds such as polyphenols, anthocyanins and falconoids are rich in fruits, vegetables, and concentrated tea and are strong inhibitors of M1 macrophage activation[57-59]. Although these compounds have been reported to be able to inhibit cancer cells by themselves, it is better to avoid taking a lot of them at the same time as Th1 response stimulation.

Inhibition of the T Regulatory System is Necessary to Obtain a Strong Th1 Response

When the Th1 response is stimulated, the T regulatory system will also be augmented. To obtain a stronger Th1 response, giving only the Th1 response stimulator is not enough; the T regulatory system needs to be inhibited.

Cyclophosphamide (CPA) is a DNA-alkylating agent that is actively against proliferating cells. CPA has been used as an anticancer drug, but its anticancer effect is not very strong. Lately, CPA has been found to be a good immune regulator, as it eliminates T regulatory cells or inhibits their activity[60]. A single low dose of CPA can induce a cytokine profile shift from Th2 to Th1 in tumor-bearing animal models[61]. Thus, it can be used to break cancer immune tolerance and enhance the function of Th1 stimulators.

In the past, some cytokines or interleukins, such as IFN-γ, IL-2, Il-12 and IL-18, have been used to directly stimulate the immune response against cancer. Among these, IL-12 is the most effective. Injection of IL-12 alone can induce complete regression of some small tumors, and the mechanism is directly related to IFN-γ production. However, injection of IL-12 alone cannot eliminate large established tumors[62,63].

Tsung *et al.*[64] first used the combination of a low dose of CPA and IL-12 to treat animals bearing large tumors. They found that if they first gave one intraperitoneal injection of 2.5 mg/mouse of CPA, and then, 4 or 5 days later, gave intraperitoneal injections of 0.5 μg of IL-12 once every other day for 5 or 9 days, large sarcomas were damaged. Mice treated with CPA and IL-12 became resistant to subsequent tumor cell challenge. Furthermore, they noted that the effect of IL-12 is based on the activation of macrophages. In IFN-γ-knockout mice, IL-12 showed little anticancer effect[65].

In 2006, Lutsick *et al.*[66] noted that a low dose of CPA actually inhibits $CD4^+CD25^+$ T regulatory cells. This

result suggested that the T regulatory system first needs to be inhibited and then, macrophage stimulators should be given. If we administer macrophage stimulators prior to inhibiting the T regulatory system, the results would be different. In CPA treatment, T cells are essential for the eradication of established tumors[67], and CPA treatment seemed to be more effective on large tumors than on small tumors.

The studies outlined in this section are quite encouraging, but sometimes the elimination of cancer by interleukin injection can cause autoimmune problems[68]. In addition, the population of T regulatory cells in cancer patients comes back about 10 days after a single low dose of CPA is given[69], suggesting that the second to the fifth day after CPA injection is the best period of time for Th1 stimulator administration.

The Combination of CPA and Oral Th1 Stimulators

It has been found recently that oral uptake of viable lactic acid bacteria will increase IL-12 and IFN-γ levels in peripheral blood[70-74]. It is possible that consuming yogurt can help cancer patients prevent recurrence of cancer[75-78]. It might be beneficial to use a combination of CPA and lactic acid bacteria to raise the blood IL-12 concentration, which would have a different result than the injection of IL-12. The injection of IL-12 will induce a high IL-12 concentration peak in the blood, but orally taking lactic bacteria will not. People have checked whether autoimmune induction occurs after orally consuming lactic acid bacteria in yogurt, and it does not[79].

In addition to lactic acid bacteria, some other edible bacteria, such as the bacteria used to make rice wine and vinegar, are also able to raise IL-12 and IFN-γ levels in the blood[80] and suppress colon cancer and B16 melanoma in mice[81].

Further Studies

In this paper, we have discussed the role of chitotriosidase in the anticancer function of macrophages (Figure 1), but the exact molecular target of chitinase on cancer cells has not yet been identified. Increasing evidence indicates that a number of proteoglycans on cancer cells are different from those on normal cells [82-84] and that tumor cells have their own types of surface mucin-type glycoproteins[85]. We believe that further studies in these areas will soon answer this question.

In addition to the stimulation of M1 macrophages in cancer treatment, recombinant human 50-kDa chitotriosidase may be directly used to treat cancer patients. If two suitable nanoparticles, one carrying recombinant human chitotriosidase and the other carrying a human protease, such as elastase[10], are prepared and intravenously injected into a cancer patient at the same time at a suitable ratio, both nanoparticles

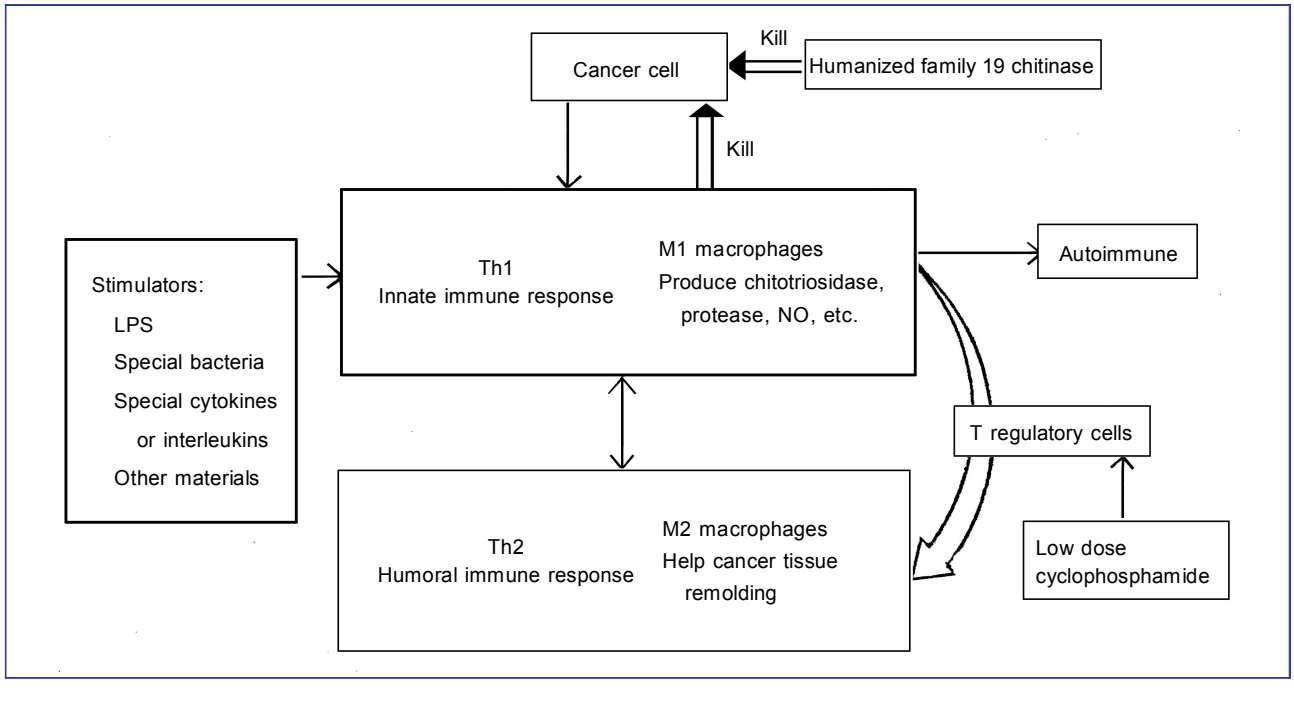

Figure 1. Schema of chitinases attacking cancer cells

will penetrate into cancer tissue through the broken blood vessels, which are present in solid tumor tissues but not in normal tissues [86]. When the enzymes are released from the nanoparticles inside the cancer tissue, the local high concentrations of both enzymes will be able to kill cancer cells.

Recently, Xu *et al.* [87] reported that a recombinant plant family 19 chitinase could kill cancer cells in culture directly. Because family 19 chitinases have a distinct structure and a catalytic mechanism different from that of family 18 chitinases, the recombinant plant family chitinase react directly and do not require protease [88,89]. However, humans do not have family 19 chitinases, though plants have many of them. Recently, two laboratories have successfully prepared humanized plants, in their study of producing humanized monoclonal antibodies, through modifying the N-glycosylation pattern of the plant [90,91]. Thus, we have the opportunity to prepare suitable humanized family 19 plant chitinases from humanized plants and use them as a new type of biochemical drug to treat cancer patients.

Acknowledgments

We are grateful to Professor Julius Kreier for his creative discussion.

Received: 2012-02-15; revised: 2012-08-30;
accepted: 2012-08-30.

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