Different activities of antitumor immunomodulators to induce neutrophil adherence response

Motoharu Tanaka¹, Shigeru Abe²,³,*

¹Department of Health and Nutrition, Faculty of Human Science, Tokiwa University; Mito, Ibaraki, Japan; ²Teikyo University Institute of Medical Mycology, Tokyo, Japan; ³Department of Sport and Medical Science, Faculty of Medical Technology, Teikyo University, Tokyo, Japan.

Summary

Functions of neutrophils, major participant in host defense mechanisms, are known to be regulated by various types of immunomodulators. Capacity of immunomodulators which are reported to show antitumor effect in vivo to induce neutrophil adherence response in vitro was investigated. Several bacterial immunomodulators (OK-432, Corynebacterium parvum, B.C.G.) and components of bacteria cell walls (lipopolysaccharide (LPS), lipid A, lipoteicic acid, N-cell wall skelton (N-CWS), muramyl dipeptide (MDP)) and fungal polysaccharides (lentinan, zymosan A, etc.) were tested. Neutrophils prepared from peripheral blood of healthy men were incubated with each immunomodulator at 37°C for 60 min in 96 well plastic plates, then neutrophils adherent to substratum were stained by crystal violet and their optical density at 570 nm was measured as a parameter of neutrophil adherence. Although purified polysaccharides mainly prepared from fungi did not induce the adherent response, not only bacterial bodies and their components but also tumor necrosis factor-α (TNF-α) clearly induced it. On the base of these results, functional classification and typing of immunomodulators by different activities in neutrophil adherence was discussed.

Keywords: Immunomodulator, neutrophil adherence, tumor necrosis factor-α, bacterial components, fungal polysaccharide

1. Introduction

Many types of immunomodulators are conceived to have antitumor activities mainly through activation of various leukocytes, such as macrophage, natural killer cells and T, and B-lymphocytes. Neutrophils have been also recognized to participate in antitumor action by bacterial immunomodulators (OK-432, etc.) and β-1,3-glucans (1-3). We reported that intraperitoneal administration of various types of antitumor immunomodulators rapidly induce neutrophils into the peritoneal cavity of mice (4). This suggested that many types of antitumor immunomodulators activate neutrophils in vivo. Neutrophils, responding to stimulus at the earliest phase in inflammatory cellular reaction, affect the following cellular reactions with host defence functions.

Clarification of antitumor immunomodulators based on neutrophil activation is important to understand their antitumor action, but it has not been studied systemically. So, we examined here the capacity of immunomodulators to activate neutrophils in vitro.

There are many parameters of neutrophil activation, such as chemotaxis, increased adherence, release of lysosomal enzymes, production of active oxygens and so on. Here, we used a neutrophil adherence assay to measure activation of human peripheral blood neutrophils because adherence to plastic plates is reported to be a reliable method for testing phagocytotic activity of neutrophils (5,6).

2. Materials and Methods

2.1. Materials

Samples were kindly donated as follows. Recombinant-human tumor necrosis factor (rhTNF); Asahi Chemical
Statistics

2.4. Statistics

Each data represents the mean of 3 values. Neutrophil adherence was observed as the absorbance at 588 nm after being stained with crystal violet, and expressed as percent value of that observed with 1 U/mL of rhTNF in the same assay plate in order to compare independent experiments.

3. Results

3.1. Time course change of LPS-induced adherence

We examined the adherence inducing ability of various biological response modifiers (BRMs) preliminary, then found that rhTNF and E. coli-LPS have potent activity on neutrophil adherence (5,6). Concentration dependency and time course change of neutrophil adherence induced by E. coli-LPS and rhTNF are indicated in Figures 1 and 2, respectively. Concentration dependent adherence was observed at 1-10^3 ng/mL by E. coli-LPS and most potent activity was seen at 60 min. On the other hand, concentration dependent adherence was observed at 10^{-1}-10^3 U/mL in the case of rhTNF. Adherence reached almost plateau level at 10 U/mL. One U/mL of rhTNF was used as the internal standard.
for all the experiments, because 1 U/mL of rhTNF induced significant neutrophil adherence constantly at submaximum level in several independent experiments (data not shown) and purified TNF can be supplied. On the incubation period, although maximum effect was seen at 120 min, submaximum effect was already obtained even at 60 min at rhTNF and maximum effect was observed at 60 min for LPS. Then, we adopted 60 min for incubation period for all the plenary studies to keep the consistency throughout the experiments.

3.2. Induction of neutrophil adherence by bacteria and its components

Neutrophil adherence activity was examined for bacteria-derived BRMs which were reported to be effective for tumor models in animals (7-15). All the tested bacteria BRMs (C. parvum, OK-432, B.C.G.) showed neutrophil adherence activities (Figure 3A).

Adherence activities of cell wall components of bacteria were indicated in Figure 3B. LPS derived from gram-negative E. coli (E. coli-LPS) induced adherence activity in a dose dependent manner. Although the adherence response by $10^{-2}$ μg/mL of LPS varied in each experiment tested, responses induced by it at more than $10^{-1}$ μg/mL seems to show relatively constant values. In the following experiments, we tested the effect of several samples for 3 concentrations or more (10 fold difference in the adjacent 2 concentrations) which had been thought to be effective in in vitro experiments.

Lipid A, which is thought to be the active center of LPS, shows a prominent effect on neutrophil even at a dose of $10^{-5}$ μg/mL which is about one tenth of that of LPS. These concentrations of lipid A and LPS on adherence correspond well with that of migrating activity which is reported by Kotani et al. (24).

Although a dose dependent increase of adherence activity was also observed in a component of gram-positive bacteria cell wall lipoteicoic acid, high concentration was necessary to show a prominent effect. Any activity was not observed in MDP even at a high dose of $10^{-2}$ μg/mL.

3.3. Effects of polysaccharides on neutrophil adherence

Neutrophil adherence activity was tested for polysaccharide-derived BRMs and some others which have been reported to be effective against tumors in animal models already (16-23) (Figure 4). Glucans such as zymosan and lentinan shows little activity even at a high dose of $10^{-2}$ μg/mL. MGA has no effect on PMN adherence also. Furthermore, TAK (linear β-1,3-glucan), which we reported to have an activating effect on neutrophil exudated in peritoneal fluid in vitro (1), did not induce adherence activity on neutrophil.
3.4. Effect of anti-rhTNF antibody on neutrophil adherence activity

Neutrophil adherence induced by bacteria and its component might be caused by some kinds of cytokines secreted from neutrophil. In such cytokines, TNF is the most probable candidates since interleukin-1 (IL-1) and platelet activating factor (PAF), which are also secreted from neutrophil when stimulated (25,26), did not show any significant adherence activities on neutrophil even at high doses (IL-1 10^3 U/mL: 18.3%, PAF 10^4 M: 25.5%, when compared with the activity of control), then we examined whether adherence activity of BRMs might be affected by the anti-rhTNF antibodies or not. Anti-rhTNF antibody (2 × 10^4 fold dilution) suppress the activity of 1 U/mL of rhTNF almost completely (2.1%, when compared with the activity of control). However, the antibody did not have any effect on the activities of 0.1 KE/mL OK-432 (without antibody: 52.5%, with antibody: 51.1%, when compared with the activity of control), 1 μg/mL E.coli-LPS (without antibody: 52.5%, with antibody: 51.1%, when compared with the activity of control) and 100 ng/mL lipid A (without antibody: 99.0%, with antibody: 98.2%, when compared with the activity of control). These data suggest that PMN adherence induced by bacteria-derived BRMs such as OK-432, E. coli-LPS and lipid A are not mediated by the TNF secreted exogenously from neutrophil.

3.5. Classification of various BRMs according to neutrophil adherence properly

General classification of BRMs according to their origin are shown in Table 1. On the right column, global classification based on the quantity of neutrophil exudate into the peritoneal cavity after injecting BRMs into mice intraperitoneally, which was reported by Morikawa et al. (4), is demonstrated. This data suggests that although most bacteria and cell wall components

in this condition (data not shown). Levamisole, a low molecular weight chemical immunomodulatory did not demonstrate any adherence activity.

Figure 4. Neutrophil adherence activities of polysaccharide-BRMs. Activity was expressed as % of that of rhTNF 1 U/mL. Neutrophil (2.5 × 10^5 cells/well) were incubated with each sample at 37°C for 60 min. Adherent neutrophils were stained and adherence activities were measured at O.D. 588nm. Each point represents the mean of 3 values. ○, lentinan; ▲, C. zymosan; ■, MGA; ●, levamisole.

Table 1. Summary of neutrophil adherence activities and neutrophil-inducing pattern of various BRMs

| Category | BRM          | Neutrophil Adherence (A) | Neutrophil induction (B) In vivo |
|----------|--------------|--------------------------|---------------------------------|
| Bacteria | G(+) B pertussis (7) | -                        |                                 |
|          | G(+) C. parvum (8)   | +                        | +                               |
|          | G(+) OK-432 (9)      | +                        | +                               |
|          | G(+) BCG (10)        | +                        | +                               |
| Component of bacteria | E.coli-LPS (11) | +                        | +                               |
|          | Lipid A (12)         | +                        | +                               |
|          | G(+) MDP (13)        | -                        | +                               |
|          | G(+) N-CWS (14)      | +                        | +                               |
| Polysaccharide | Lipoteicoic acid (15) | +                        | +                               |
|          | MGA (16)             | -                        |                                 |
|          | Carrageenan (17)     | -                        | +                               |
|          | Dextran sulfate (18) | -                        | +                               |
|          | Lentinan (19)        | -                        | +                               |
|          | β-1,3glucan (20)     | -                        | +                               |
|          | Zymosan A (21)       | -                        | +                               |
| Others   | Levamisol (22)       | -                        |                                 |
|          | Poly(I)-poly(C) (23) | -                        | +                               |

BRMs showing in vivo antitumor effect are categorized based on their origin, and neutrophil inductive activity in vivo and neutrophil adherence activity in vitro are indicated. G(-); gram negative, G(+) ; gram positive. (A) Relative activities of neutrophil adherence were classified according to their potencies. +, adherence more than 50% of that of rhTNF 1 U/mL was observed at 10 μg/mL, and dose dependency was observed. -, No adherence activity was observed even at 10^2 μg/mL or 10^4 cell/mL. (B) Quoted from the paper of K. Morikawa et al. (4). ++, Highly potent type inducing more than 6 × 10^6 neutrophil/mouse; +, Relatively low neutrophil-inducing type inducing less than 6 × 10^6 neutrophil/mouse; -, No neutrophil-inducing activity. In vivo antitumor activities on each BRM are shown below.
of bacteria-derived BRMs have neutrophil adherence activity, polysaccharide and another BRMs (levamisole, poly (I)-poly (C), ET-18-OMe) do not have any such property in vitro although induction of PMN into peritoneal cavity are observed in vivo (16-23).

4. Discussion

In this report, we examined direct neutrophil adherence inducing properties of various antitumor BRMs. All the BRMs tested in this study are reported to have antitumor effect in vivo (7-23). Adherence activities of neutrophil to plastic plates were found in E. coli-LPS, N-CWS, bacteria and its components such as lipid A and lipoteichoic acid. In summary, bacteria-derived BRMs have direct activating properties on neutrophil. On the other hand, antitumor polysaccharide such as lentinan, zymosan and β-1,3-glucan, which show antitumor activity, did not show adherence induction on neutrophil at all.

We reported already that all of the BRMs tested in this paper (except levamisole, dextran sulfate) induce neutrophil more than 10⁶ cells/mouse into peritoneal cavity within 6 h after injection (4). These data suggest that these BRMs must have some influence on neutrophil in the body. Considering from these data and our new data that most bacteria-derived BRMs except B. pertussis and MDP can induce the adherence of neutrophil and polysaccharide do not effect on neutrophil adherence at all, bacteria-derived BRMs must have an effect on neutrophil directly, but another ones (polysaccharides) must accumulate neutrophil indirectly through the activation of complements or macrophage in vivo. For example, zymosan is well known to activate neutrophil prominently by activating complement of alternative pathway (27). However, in this system, complement system does not work, because only inactivated bovine serum was used. Lack of complement system must be the reason why zymosan did not induce neutrophil adherence.

Another different point is such BRMs that directly activate the neutrophil have local antitumor effects generally (12,28). In other words, such BRMs are effective prominently, if they are injected locally to contact with tumor tissue. On the other hand, such agents as polysaccharides and poly(I)-poly(C), which did not induce neutrophil adherence directly, are not reported to have local antitumor activity (28). This relationship suggests that local antitumor effect of bacteria-derived BRM might be connected with direct activation of neutrophil and systemic antitumor effect of polysaccharide might be connected with indirect activation of neutrophil through the activation of complements or macrophages.

If TNF producing ability of BRMs are classified into 2 groups (priming agents and triggering agents), BRMs that induce the adherence of neutrophil directly (OK-432, LPS, lipoteichoic acid et al.) are known to be TNF triggering agents (29-32). On the other hand, another BRMs such as MDP, glucan and zymosan are reported to have priming activity of TNF release (30,33). Considering from these facts and the report that neutrophil can produce TNF in certain condition (34), direct induction of PMN adherence by bacteria-derived BRMs is considered to be caused by TNF released from neutrophil. Then, we tested the effect of rhTNF antibody on neutrophil adherence induced by BRMs. However, induction of neutrophil adherence by LPS, lipid A and OK-432 were not influenced at all. Then it is improbable that TNF secreted from neutrophil by BRM mediate the adherence of neutrophil.

It is reported that increased activity of neutrophil by LPS depends on the increased expression of CD11b/CD18 via TLR-4 (35,36). On the other hand, TLR-2 is thought to mediate responses to Gram-positive bacterial protein (37). TLR-2 as well as TLR-4 are expressed in neutrophils in addition to macrophages (38). Then, stimulated adherence by E. coli LPS, lipid A and OK-432 may be explained by the increased expression of CD11b/CD18 through TLR4 and the increased adherence by N-CWS and lipoteichoic acid may be mediated by TLR-2 expressed in neutrophils in addition to macrophages. As activation of TLR4 and TLR2 are reported to be tumoricidal (11,39), antitumor mechanism of bacterial BRMs may depend on the activation of TLR4 and/or TLR2 expressed in neutrophil in addition to macrophage. So far, the mechanism of antitumor effect of BRM was explained mainly through the activation of macrophage (40). However, neutrophils and macrophages are considered to coordinate in immune response in several diseases (41). Therefore, neutrophils activated by bacterial BRMs may attack tumors directly and/or indirectly in cooperation with macrophages.

In conclusion, direct activation of neutrophil may be related with the local antitumor effect of bacteria-derived BRM to some extent.

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