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ISOLATION, CHARACTERIZATION AND EVALUATION OF SKIN PEPTIDE ISOLATED FROM INDIAN BULL FROG *RANA TIGRINA*

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ABSTRACT
It has been observed that the normal functioning of the immune system is generally impaired in certain infections as well as tumor-bearing hosts. Some infections like HIV and tumor cells have been shown to suppress the immune response either directly or by activating the host's suppressor mechanism. Spontaneously originating transplantable tumor of T cell origin, designated as Dalton's lymphoma (DL) generally have very low immunogenicity and able to evade the immune system of the host. Skin peptides isolated from a number of frog species have been found to boost the mammalian immune system to fight against some infections and tumor conditions. In the present study, the active peptide has been isolated from the skin of *Rana tigrina* and characterized. The purified protein has molecular weight of approximately 3.2 kD as evident from SDS-PAGE analysis. IL-1 and TNF assay have been performed to evaluate the skin peptide. After treatment with frog peptide significant increase in the level of IL-1 and TNF production have been observed in both normal as well as tumor associated (DL) macrophages of experimental mice, which indicates that the peptide has the potential to be used as drug for the treatment of tumors and infections as these cytokines play crucial roles in cell mediated immunity.

KEY WORDS: Frog peptide, Dalton's lymphoma, Inter Leukin-1, *Rana tigrina*, Tumor Necrosis Factor-α.

INTRODUCTION

In vertebrate immune system, macrophage plays an important role in innate and adaptive immune responses. Macrophages have been considered to be first line of defense mechanism against infection and malignancies in the host (Morimoto and Santaro, 1998; Kaufmann, 1990). Upon activation, it produces various specific and non-specific effector molecules including pro-inflammatory cytokines and chemokines necessary for recruitment of lymphocytes at the site of antigen entry or tumor progression and show profound bactericidal and tumoricidal activity (Mosser, 2003). It has been found that tumor cells are able to suppress the function of macrophages and skews it to M2 phenotype or alternatively activated macrophage population in the tumor-bearing host. Therefore, in the tumor-bearing host, the cytotoxic functions of macrophages get altered, which invariably results into tumor development and progression. Skin secretions from frogs belonging to the family Ranidae (''true frogs'') have proved to be a particularly rich source of antimicrobial peptides and more than 200 such peptides from approximately 60 species have been described (Conlon *et al.*, 2004 and 2009). Indian bull frog *Rana tigrina* is most abundant species of frog in Indian continent. Therefore, there is a great need to isolate, purify and characterize antimicrobial peptides of *Rana tigrina* as it could be a cheap source of antimicrobial/tumoricidal peptide and it could be made commercially available for therapeutic use.

The important goal of this study was to determine whether skin peptides from *Rana tigrina* could activate macrophages in tumor bearing mice to inhibit growth of tumor progression. In the present study, the active peptide has been isolated from the skin of *Rana tigrina* and lyophilized. SDS-PAGE analysis has been done for the characterization of the skin peptide. The purified protein has molecular weight of approximately 3.2 kD. IL-1 and TNF assay have been performed to evaluate the skin peptide. After treatment with frog peptide significant increase in the level of IL-1 and TNF production have been observed in both normal as well as tumor associated (DL) macrophages of experimental mice, which indicates that the peptide has the potential to be used as drug for the treatment of tumors and infections as these cytokines play crucial roles in cell mediated immunity.

MATERIALS AND METHODS

All the chemicals were obtained from Sgma-Aldrich Chemical Private Limited, India. Indian bull frog *Rana tigrina* were collected from local vicinity of Varanasi for extraction of the Skin peptides. Inbred strains of healthy BALB/c ($H2^d$) mice of either sex at 8 – 10 weeks of age were used for the experiments. T-cell lymphoma of spontaneous origin –Dalton lymphoma (DL) was used as tumor model. Frog peptides were isolated by a standard method described by Amanda *et al.*, 2001, estimated by Lowry method (Oliver *et al.*, 1951) and resolved in 15% SDS-PAGE together with molecular weight marker (M 3546, Sigma-Aldrich).

Preparation of different doses of skin peptides

Lyophilized skin extract was solubilized in triple distilled water at the concentration of 10 mg/ml. This stock solution was further diluted in various concentrations of 60, 80, 100, 200, 400 and 600 µg/ml.

Macrophage isolation

Mice with and without tumor were killed by cervical dislocation and peritoneal exudates cells (PECs) were harvested by peritoneal lavage using chilled serum free RPMI 1640 as described by Sodhi *et al*., 1992.

IL-1 activity was assayed by a standard thymocyte proliferation assay as described by Mizel, 1982 and TNF assay was performed in cell free culture supernatant of treated (4 µg/ml LPS, 100 µg/ml peptide & LPS + peptide) or untreated macrophages collected after 24 hours by standard cytotoxicity assay against TNF sensitive cell line L929 as described by Flick and Gifford, 1984 using the formula:

$$\% \text{ Cytotoxicity} = \frac{C - E}{C} \times 100$$

Where, C = Control
E = Experimental cells

RESULTS

Sds-page of skin peptides

Samples of frog's skin peptides were resolved in 15% SDS-PAGE together with molecular weight marker (M 3546, Sigma-Aldrich) and stained in Coomasie blue stain and scanned. SDS-PAGE of peptides shows a single major peptide band of approximately 3.2 kDa as evidenced from Figure 1.

Effect of the skin peptides on macrophage activation

We have here measured IL-1 and TNF production on proper stimulation of peritoneal macrophages from both normal and DL-bearing mice. The result has been compared with double control; untreated (negative) and LPS treated (positive) as a standard macrophage activating agent for macrophage activation.

Interleukin-1 (il-1) assay

It has been found that the production of IL-1 in macrophages treated with LPS and peptides shows enhanced production of IL-1 in comparison to macrophages treated with medium alone as shown in Fig. 2. The augmentary effect of peptides on the production of IL-1 in tumor-associated macrophages has been observed. TAMs show the production of IL-1 which indicates that peptides has augmentary effect not only on the production of IL-1, but also on the proliferation and differentiation of the two main subsets of T-lymphocyte ($CD4^+$ and $CD8^+$ T-lymphocytes) in which the tumor bearing host, due to immunosuppressive activity of DL- cells, the number is depleted. Therefore, it is assumable that peptides treatment may help in the restoration of T-lymphocytes counts in the tumor bearing host.

Tumor necrosis factor (tnf) assay

The incubation of normal macrophages with LPS has been taken as a positive control for the production of most potent cytokine for the killing of tumor cells, TNF-α. As result shows in Fig. 3, that the macrophages treated with peptides or in combination with LPS shows the enhanced production of TNF-α. The percentage increase in cytotoxicity against the killing of L929 cells indicates that significant amount of TNF-α is produced by macrophages upon proper stimulation with peptides. Further, it has been reported that the TAMs also be activated by the peptides. But normally the TAMs do not show the tumoricidal activity because, as the tumor progresses, the phenotype of macrophages are changed to M2 phenotype also named as alternatively activated macrophages that invariably helps in tumor progression. Therefore, it might be possible that peptides have a potential to revert the M2 phenotypes of macrophages into M1 phenotype for the effective killing of the tumor cells.

DISCUSSION

Classically, the immune system was separated into two branches: humoral immunity, for which the protective function of immunization could be found in the humor (cell-free body fluid or serum) and cellular immunity, for which the protective function of immunization was associated with cells. CD4 cells or helper T cells provide protection against different pathogens. T cells cause death by apoptosis without using cytokines; therefore in cell mediated immunity cytokines are not *always* present.

Cellular immunity protects the body basically in three ways:

1. By activating antigen-specific cytotoxic T-lymphocytes that are able to induce apoptosis in body cells displaying epitopes of foreign antigen on their surface, such as virus-infected cells, cells with intracellular bacteria, and cancer cells displaying tumor antigens;
2. By activating macrophages and natural killer cells, enabling them to destroy pathogens; and
3. By stimulating cells to secrete a variety of cytokines that influence the function of other cells involved in adaptive immune responses and innate immune responses.

Cell-mediated immunity is directed primarily at microbes that survive in phagocytes and microbes that infect non-phagocytic cells. It is most effective in removing virus-infected cells, but also participates in defending against fungi, protozoans, cancers, and intracellular bacteria. It also plays a major role in transplant rejection.

The findings of present research suggest that the frog peptides isolated from *Rana tigrina* directly or indirectly induce the production of cytokines, IL-1 and TNF-α in normal and altered macrophages (TAMs). *In vivo* treatment with the peptides showed enhanced production of IL-1 and TNF-α by TAMs. Macrophages are considered as the first line of defense in conferring innate immunity against infection and malignancies. However, tumor growth simultaneously modulates their functions (Ohashi *et al.,* 2000), and as a result they lose their normal functions and eventually have tumor protective function. Usually, TAMs produce several cytokines of immunosuppressive function or tumor growth promoting function, such as IL-10 and TGF-β and other suppressive mediators such as prostaglandin. In the present study higher production of ilterleukin-1(IL-1) and tumor necrosis factor (TNF-α) was observed by TAMs after treatment with frog peptides in DL-bearing mice. Therefore, the release of these cytokines IL-1and TNF-α are considered as major tumoricidal action of activated macrophages *in vitro* (Hibbs *et al.,* 1988; Stuehr *et al.,* 1989; Cui *et al.,* 1994) and *in vivo* (Yim *et al.,* 1993; Farias-Eisner *et al.,* 1994).

Previously, the pathophysiological relevance of the TAMs in response to either microbial or biological response modifiers (BRMs) was lacking in any tumor cell types including T cell lymphoma. TAMs have different characteristics than their normal counterpart due to association with tumor. Since, results confirmed the enhanced activity of TAMs upon activation by frog peptides; it can be assumed that this increased activity of TAMs is due to reversal of their altered function towards normal which could be relevant in the formulation of immunotherapeutic protocols against malignancies. Normally, most of the BRMs that are constitutively synthesized and localized in the cytosol; its expression is induced in inflammatory conditions (Soltys *et al.,* 1996 and 1997; Laplante *et al.,* 1998) such as in malignancies and other infectious diseases. Necrosis of these inflammed cells results in the higher local concentration of certain factors including Hsp70 in extra cellular microenvironment which stimulates macrophages and other immune cells and triggers the immunologically relevant genes (Deepak *et al.,* 2006). Un-stimulated macrophages do not show any changes at synthesis level as well as in the production of cytokines. Therefore, it can be concluded that peptide treatment must exert stimulatory effect at the level of transcription and expression of the immunologically relevant genes.

Result shows that *in vivo* and *in vitro* treatments of frog peptides activate TAMs in a manner similar to normal resident macrophages and enhances the production of inter- leukins. Likewise, the enhanced production of IL-1 by TAMs indicated that frog peptides could revert back the normal functioning of TAMs even in the tumor microenvironment of DL-bearing mice. It has been further observed that following exogenous application; frog peptides increase LPS responsiveness in DL-bearing mice.

Recognizing that tumor growth negatively regulates macrophage function in production of immunomodulatory soluble factors, it followed those therapeutic agents that reconstitute immune activity through indirect action by inducing the production of stimulatory cytokines. Prior treatment or immunization of tumor bearing host (TBH) with frog peptides reverses the suppressed activities of TAMs to normal one. Therefore, it is suggested that frog peptides might enhance T cell reactivity by enhancing IL-1 production (Chaly *et. al.*, 2000), which subsequently changes the antigen presenting function of TAMs *in situ* that in turn are able to induce clonal selection and expansion of naïve T cells and their effector function. Results also suggest that frog peptides treated TAMs induce T cell proliferation. Here, it is interesting to note that treatment of TAMs with LPS comparatively could not achieve such parameters as well. However, LPS treatment of NMO produces higher amount of IL-1β than TAMs which confirms this assumption that NMO is more responsive towards LPS than TAMs. The differences observed in IL-1β production on treatment with frog peptides *in vitro* and *in vivo* condition was possible due to endogenous environment provided by host and interdependent stimulation of each factors.

This study demonstrates anticancer activity of skin peptides isolated from Indian bull frog *Rana tigrina*. We report the isolation, characterization and immunomodulatory potential of frog peptide with an exceptionally large spectrum activity against cancer. In this study, we have shown that the peptides derived from the skin of *Rana tigrina* are capable to restore not only the suppressed function of TAMs, but also enhance the production of various effector molecules and proinflammatory cytokines.

These factors together, make the immune system more compatible to suppress infections and tumor growth effectively, in the host mice and thus enhance the survival of tumor-bearing host. It has been shown that *in vitro* and *in vivo* treatment of frog peptides induces production of these non-specific effector molecules, both by normal macrophages (NMO) and TAMs.

Legends

SDS-PAGE ANALYSIS OF FROG SKIN PEPTIDE

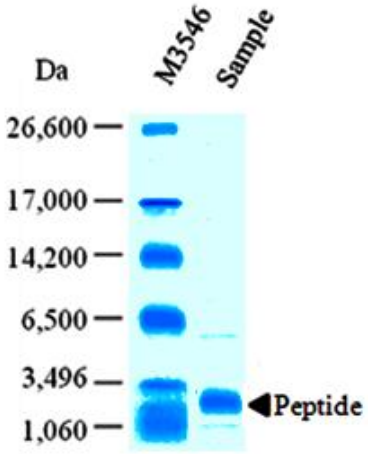

Figure 1. SDS-PAGE of skin peptides.

Interleukin-1 (IL-1) assay

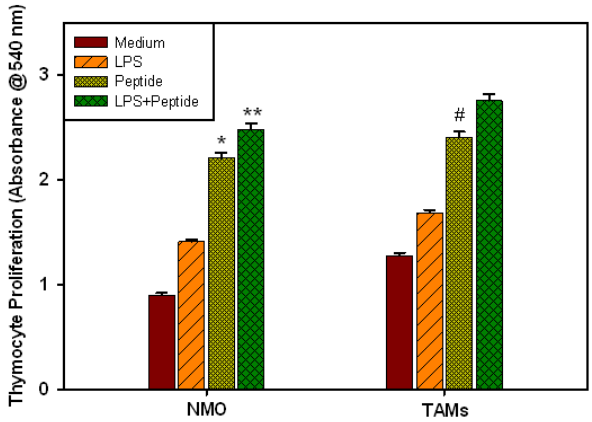

Figure 2. Effect of skin peptide on IL-1 production.

Tumor necrosis factor (TNF) assay

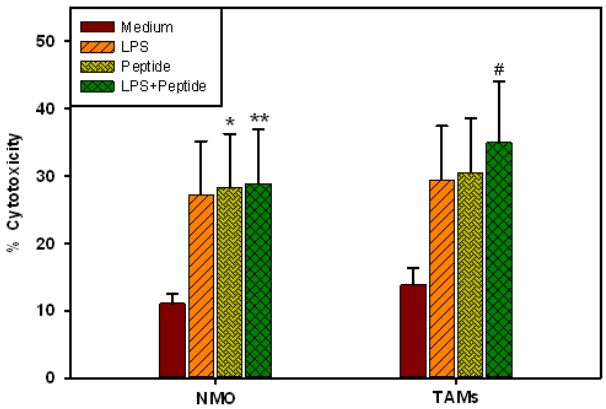

Figure 3. Effect of skin peptide on TNF-α production.

CONCLUSION

Present study shows that skin peptides isolated from *Rana tigrina* directly induces the production of a number of non-specific effector molecules like IL-1 and TNF-α by normal and altered macrophages which are necessary to kill tumor cells. However, tumor growths simultaneously modulate the function of macrophages residing in their close vicinity.

As a result, tumor-associated macrophages (TAMs) lose their normal functions and secrete very less amount of these effector molecules. Usually, tumor- associated macrophages produce fewer amounts of cytokines, such as IL-1 and TNF-α, but results show that skin peptides treatment restores their normal phenotype and function. It has been shown that *in vitro* and *in vivo* treatment of frog skin peptides induces higher production of these non-specific effector molecules, both by normal macrophages (NMO) and TAMs.

On the basis of these observations, it is concluded that the frog peptides have the capability to activate various immune cells directly or indirectly. The peptides derived from the skin of *Rana tigrina* are capable to restore not only the suppressed function of TAMs, but also enhance the production of various effector molecules and proinflammatory cytokines.

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