VI - SCAVENGING ACTIVITY OF MACROPHAGE

6.1. INTRODUCTION

Macrophage (Greek: big eaters, from macros “large” + phagein “eat”): are white blood cells produced by the differentiation of monocytes in tissues. Macrophages function in both non-specific defense (innate immunity) as well as specific defense mechanisms (adaptive immunity). Their role is to phagocytose (engulf and then digest) cellular debris and pathogens, either as stationary or as mobile cells (Adams 1992). They also stimulate lymphocytes and other immune cells to respond to pathogens. These specialized phagocytic cells attack foreign substances, infectious microbes and cancer cells through destruction and ingestion. One important role of the macrophage is the removal of necrotic cellular debris in the lungs. Removing dead cell material is important in chronic inflammation, as the early stages of inflammation are dominated by neutrophil granulocytes. When a macrophage ingests a pathogen, the pathogen becomes trapped in a phagosome, which then fuses with a lysosome. Within the phagolysosome, enzymes and toxic peroxides digest the pathogen. Macrophages survive longer in the body up to a maximum of several months.

The concept that phagocytic cells play a role in the defense of the organism against foreign intruders was first elaborated by Metchnikoff (1901), who subsequently claimed that macrophages are activated in response to an inflammatory challenge (Metchnikoff 1905). Such activated macrophages were found to exhibit a high antitumor cytotoxic response (Ruco and Meltzer 1978). Macrophages have three major functions in inflammation, antigen presentation, phagocytosis, and immunomodulation through production of various cytokines and growth factors. Macrophages play a critical role in the
initiation, maintenance, and resolution of inflammation. They are activated and deactivated in the inflammatory process. Tumour necrosis factor (TNF) is a major mediator of inflammation, with actions directed towards both tissue destruction and recovery. While inducing death of diseased cells at the site of inflammation, TNF can also destroys tumour blood vessels (Lejrune et al., 1998).

Macrophages are versatile cells that play many important roles. As scavengers, they remove the body of worn-out cells and other debris and as secretory cells, macrophages are vital to the regulation of immune response and the development of inflammation. Inflammation known to contribute to cancer development and progression, and the association between these two processes has been more important. A number of chronic inflammatory diseases have been shown to be associated with a variety of cancers. (Balkwill et al., 2005; Balkwill et al., 2001; Coussens and Werb 2002; Mantovani 2005). Cancer associated inflammation includes the infiltration of white blood cells, phagocytic cells called tumour associated macrophages (TAM) (Matovani et al., 2002); cytokine such as tumour necrosis factor (TNF) or interleukin-1 (IL-1), chemokine etc.

The biological activity of macrophages is mediated by functionally distinct subpopulations of macrophages that are phenotypically polarized by their microenvironment and by exposure to inflammatory mediators. These divergent macrophages are broadly classified into classically activated M1 macrophage and alternatively activated M2 macrophages. M1 macrophages are activated by type- I cytokines like interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), or after recognition of pathogen associated molecular patterns or PAMPs (lipopolysaccharide, lipoproteins, lopolteicoic acid) and endogenous “danger” signals. M1 macrophages exhibit potent microbicidal activity, and release (interleukin) IL-12, and IL- 23 (Verreck et
combine with low levels of IL-10, promoting strong Th1 immune responses (Van Ginderachter et al., 2006). In addition, they exert anti-proliferative and cytotoxic activities, which is due in part to the release of reactive oxygen and nitrogen species (NO, peroxynitrite, hydrogenperoxide, superoxide) and proinflammatory cytokines (TNFα, IL-1, IL-6) (Urban et al., 1986; Stuehr et al., 1989; Mytar et al., 1999; Bonnotte et al., 2001; Edward et al., 2006; Van Ginderachter et al., 2006).

Alternatively activated M2 macrophages are further subdivided into M2a (activated by interleukin (IL)-4 or IL-13), M2b (activated by immune complexes in combination with IL-1β or LPS) and M2c (activated by IL-10, transforming growth factor-β or glucocorticoids). M2 macrophages release IL10 and exert selective immunosuppressive activity, and inhibit T-cell proliferation, play a role in the resolution of inflammation through phagocytosis of apoptotic neutrophils, reduced production of pro-inflammatory cytokines, and increased synthesis of mediators important in tissue remodelling, angiogenesis, and wound repair (Bingle et al., 2002; Gordon 2003; Mantovani et al., 2004; Sica et al., 2006;).

Macrophages are capable of both antibody-dependent and independent phagocytosis of tumor cells (Silverstein et al., 1989). Macrophages have long been postulated to play a role in host defense against malignancy although the exact nature of the interaction between tumor cells and macrophages has not been fully defined. One way that macrophages may kill tumor cells is through ADCC (antibody-dependent cell-mediated cytotoxicity).

Bucana et al., (1976) reported that the killing of tumour cells by macrophages involves direct contact between macrophages and tumour cells and also showed morphological evidence that in direct contact between the macrophages and the tumour
cells lysosomal organelles are translocated from cytotoxic macrophages into the cytoplasm of target tumour cells and destruction of target tumour cells by activated macrophages is a nonphagocytic, lytic process, occurring during contact between the target cells and the activated macrophages. Binding of macrophages to tumour cells is an essential step in the process of cytolysis and the unstimulated peripheral blood monocytes express cytolytic activity against melanoma cells and that human monocytes and alveolar macrophages were cytotoxic toward tumour cells without prior activation.

The better characterized response of macrophages to microbial molecules, cancer cells, and host cytokines is the release of inflammatory/microbicidal/tumoricidal products. Macrophages secrete a great many products, enzymes, complement components, reactive oxygen intermediates, arachidonic acid intermediates, coagulation factors, cytokines and other factors including nitrite as a final compound of the L-arginine metabolic pathway. Among these secretory products, arachidonic acid intermediates (Prostaglandins and leukotrienes), cytokines, and nitrite are deeply involved in expression of antitumour activity.

Macrophages have well-documented functions in the regulation of the antitumour immune response. As the presence of a growing tumour is associated to stromal remodelling and to the production of proinflammatory molecules that act as danger signals. MI macrophages, as well as (natural killer) NK cells and DC (dendritic cells), are thus attracted into the injured site, resulting in a massive secretion of IFN-γ and IL-12 ((Boehm et al., 1997; Munder et al., 1998; Harris et al., 2000; Ibe et al., 2001; Tsung et al., 2002; Brigati et al., 2002). Indeed, the products generated during the stromal remodelling induce macrophages to produce IL-12, which stimulates NK cells to produce IFN-γ, which in turn activates macrophages to produce more IL-12 leading to a positive feedback increasing the
IFN-γ production by NK cells (Bancroft et al., 1991). In addition, a newly described subset of DC, called IKDC, contributes to IFN-γ. MI macrophages release tumoricidal products, such as reactive oxygen intermediates and NO, which kill tumour cells. (Nathan 1987; MacMicking 1997). As a result, tumour antigens from dead tumour cells become available, and the adaptive immune system is recruited. Activated macrophages also produce TNF-α which can kill tumour cells by direct effect on tumour cells or by inhibiting the developing vasculature (McBride 1986; Blankenstein et al., 1991). Finally M1 macrophages, which are APC (antigen presenting cells), process tumour antigens and present them to lymphocytes after migration into the lymph nodes. As a result, the different subsets of T lymphocytes are activated, proliferate, and thus infiltrate the tumour, where they can exert their immune function. (Dunn et al., 2004).

The use of macrophages against tumour cells have several advantages over use of other types of leukocytes such as natural killer cells and antitumor-specific T&B lymphocytes; Antitumour activity of macrophages is very wide and is not species specific. Human macrophages can react against murine tumor cells (Ben-Efraim et al., 1991) and murine macrophages can be induced to react against human tumor cells (Hibbs 1974). Activated macrophages can react indiscriminately against “immunogenic” “weakly immunogenic” and “non immunogenic” tumors (Hibbs et al., 1972; Fidler 1985) Non active resident macrophages can be activated in vitro to express antitumor activity and human peripheral blood monocytes can be matured in vitro (Andreesen et al., 1988; Dummont et al., 1988) and induced to react against tumor cells. Macrophages and human peripheral blood monocytes can easily be isolated from cancer patients, activated in vitro, and reinjected in the same donor patients. Clinical trials based on this principle have been reported (Andreesen et al., 1990; Bartholenys et al., 1991)
Macrophage activation is in fact a dynamic process; thus the same cells may initially take part in proinflammatory and cytotoxic reactions and later participate in the resolution of inflammation and wound healing (Porcheray et al., 2005; Benoit et al., 2008). This suggests that macrophages undergo progressive functional changes as a result of alterations in their microenvironment (Stout et al., 2004; Edward et al., 2006; Martinez et al., 2008). The inflammatory environment enhances the antitumour cytostatic activity of human macrophages, by the secretion of antitumour cytokines into the medium or cell to cell contact..

Macrophages participate in tumour cell killing through varied processes, the release of soluble factors, tumour necrosis factor, lysosomal enzymes and phagocytosis. The induced phagocytic activity by sepiia and squid ink was studied by several researchers. Liu (1993); Lu et al., (1994); Lu et al., (1995) studied the inducing effect sepiia ink on macrohages; Sasaki et al., (1997) studied the enhanced phagocytic activity of squid ink; Lu et al., (1999) studied the squid ink extract on macrophages; He.S et al., (1999, 2003) studied the macrophage activation of sepiia ink; Masayo et al., (2000) reported activation of macrophages by squid ink in curing cancer; Xie et al., (2001, 2002), studied the inducing effect of sepiia ink of colony stimulating factor. Previous studies (Chapter V) demonstrated the anticancer activity of the ink and bone of squid and cuttle fish. The main objective of this scavenging assay is to investigate the activation of the extracts (ink, bone) from squid and cuttle fish on macrophages in vitro in order to understand further the mechanism of extracts against cancer cells.

6.2. MATERIALS AND METHODS

Macrophage scavenging assay is otherwise known as NBT (nitroblue tetrazoliun) reduction assay. NBT assay is used to determine the production of superoxide anion (\(O_2^-\))
in various phagocytic cells. This microscopic assay is conducted by counting the cells containing blue NBT formazan deposits, which are formed by reduction of the membrane permeable, water-soluble, yellow-coloured, nitroblue tetrazolium by O$_2^-$. Macrophage scavenging was performed with RAW 264.7 cell line (murine macrophage cell line) obtained from National centre for cell science, Pune, India.

The NBT reduction assay was carried out as previously described (Rainard, 1986). Briefly, 20µl of the macrophage suspension and 40 µl of RPMI medium (Roswell Park Memorial Institute) were added in a flat bottom 96-well plate (Nunc®, USA). Twenty microliter of the solution containing the crude ink, fractioned (30% & 80%) protein ink and methanol extract of bone powder dissolved in 0.1% dimethylsulfoxide (DMSO) in phosphate buffer saline (PBS) solution was added in each well to give final extract concentrations of 10, 100, 500 and 1000 µg/ml. The 0.1% DMSO in PBS (without the extract) was used as a control. After incubation for 24 h at 37$^\circ$C in 5% CO$_2$ humidified atmosphere, 20µl of the heated inactivated yeast (Candida albicans) suspension (5×10$^7$ particles/ml) and 20 µl of NBT (Sigma, Germany) solution in PBS (1.5 mg/ml) were added and the mixture was further incubated under the same conditions.

After incubation for 60 min, the adherent macrophages were rinsed vigorously with RPMI medium and washed four times with 200 l methanol. Air-dried, 120 µl of 2M KOH and 140 µl of DMSO were added. The absorbance was measured at 570 nm by a well reader (Biorad Plate reader) and the percentage of NBT reduction was calculated by the following equation

\[
\text{NBT reduction} \, (\%) = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD negative control}} \times 100
\]
The EC50 value represents the effective concentration required for 50% enhancement of oxidative burst reduction activity.

6.3. RESULTS

Macrophage scavenging activity of extracts (ink and bone) from *Loligo duvauceli* and *Sepia pharaonis* are presented in Plate. 23, Figs. 32-35.

6.3.1. MACROPHAGE SCAVENGING ASSAY FOR CRUDE *L. DVAUCELI* INK:

Crude squid ink protein showed varied activity ranging from 13% to 75%. (Fig.33). At a concentration of 10µg/ml crude ink showed 13% of scavenging activity. The macrophage scavenging activity was increased as the concentration of ink increased and the activity raised to 75% at 1000µg/ml was subjected. Scavenging activity was higher at higher concentration of crude ink (protein).

6.3.2. MACROPHAGE SCAVENGING ASSAY FOR FRACTIONATED 30% *L. DVAUCELI* INK:

Fractioned 30% squid ink protein was subjected to macrophage scavenging assay at varied concentrations of 10, 100, 500 and 1000µg/ml.(Fig.32) The macrophage scavenging was observed in all concentrations of proteins ranging from 5% to 82% in which 10µg/ml concentration of protein showed 5% of activity followed by 58, 72 and 82% of activity was observed in the increased concentration (1000µg/ml) of protein. Macrophage activity of 30% fractioned protein showed higher percentage of activity when compared to crude protein.
6.3.3. MACROPHAGE SCAVENGING ASSAY FOR CRUDE S. PHARAONIS INK:

Protein from cuttle fish ink showed varied activity ranging from 2% to 60%. (Fig.33). At a concentration of 10µg/ml of crude protein showed 13% of scavenging activity. The macrophage scavenging activity was increased as the concentration of crude protein was increased. The activity was raised to 60% when 1000µg/ml of protein was added. Scavenging activity was higher at 1000µg/ml of crude protein.

6.3.4. MACROPHAGE SCAVENGING ASSAY FOR FRACTIONATED 80% S. PHARAONIS INK:

Fractioned 80% protein was subjected for macrophage scavenging assay. (Fig.34). The proteins were subjected at varied concentrations of 10, 100, 500 and 1000µg/ml. The macrophage scavenging was observed in all concentrations of proteins varied from 4% to 65% in which 10µg/ml concentration of protein showed 4% of activity followed by 47, 59 and 65% of activity in the increased concentration of protein. Macrophage activity of 80% fractioned protein showed higher percentage of activity when compared to crude protein.

6.3.5. MACROPHAGE SCAVENGING ASSAY FOR L. DUVAUCELI BONE POWDER:

Bone powder of squid was subjected to solvent extraction to separate the active bio compounds and the compounds extracted were tested for macrophage scavenging assay. The compounds were treated at varied concentrations of 10, 100, 500 and 1000µg/ml and the activity varied from 5% to 16%. Highest activity was observed at the concentration of 1000µg/ml and the activity was increased as the concentration of bioactive compounds increases. Highest activity of 16% was observed at 1000µg concentration of bioactive compounds. (Fig.35)
6.3.6. MACROPHAGE SCAVENGING ASSAY FOR S.PHARAONIS BONE POWDER:

Bone powder of cuttle fish was subjected to solvent extraction and the separated active bio compounds were subjected to macrophage scavenging assay. The compounds was treated at varied concentrations of 10, 100, 500 and 1000µg/ml and the activity varied from 4% to 15.5%. Highest activity was observed at a concentration of 1000µg/ml. The activity was increased as the concentration of bioactive compounds increases. Highest activity of 15.5% was observed at 1000µg concentration. (Fig.35)

6.4. DISCUSSION

Macrophages are mononuclear phagocytes derived from bone marrow precursors and differentiate into monocytes which circulate in the blood. The majority of monocytes localize in tissues and mature into macrophages where they develop specialized functions depending on the needs of the tissue. Macrophages perform a multitude of functions essential for tissue remodeling, inflammation and immune response against cancer (Bingle et al., 2002)

In the present investigation crude ink, fractioned protein ink and methanol extracts of bone powder from squid Loligo duvaucelii and cuttlefish Sepia pharaonis has been tested against RAW 264.7 macrophage cell line because in vitro, Phagocytosis is an important mechanism of macrophage anti-tumour cytotoxicity, since human tumour cells are relatively insensitive to the better-known mediators of macrophage killing such as tumour necrosis factor and reactive oxygen intermediates. The macrophage scavenging activity has increased as the concentration of crude ink was increased. The activity was 8% at 10µg/ml concentration and raised to 75% at 1000µg/ml of concentration of squid ink.
Fractioned protein squid ink (30%) showed minimum (12%) macrophage scavenging activity at 10µg concentration and showed highest (82%) at 1000µg/ml concentration. Macrophage activity of 30% fractioned protein of squid ink showed higher percentage of activity when compared to crude protein.

The induced phagocytic activity by squid ink was studied by several researchers. Sasaki et al., (1997) studied the anti-tumour activity of the acetone extraction of squid ink. The delipidated ink enhanced the phagocytic activity of macrophages and the antitumour activity of the delipidated ink was mainly due to the cellular immunity in vivo. Lu et al., (1999) investigated the activation of the extracts from squid ink on macrophages in vivo. The levels of IL-I and TNF α in the animals treated with the squid ink extracts were significantly increased and they concluded that the antitumor effects could be indirectly induced by squid ink through its phagocyte activation. Masayo et al., (2000) reported that the mucopolysachharide peptide complex in squid ink have the effect of curing cancer by the activated macrophages. Guan Ling et al., (2010) reported that the squid ink plays a critical role in recovery of the cellular immunity in immune suppression mice.

In the present study highest scavenging activity of 60% was observed at 1000µg/ml concentration of crude cuttle fish ink and the activity of 70% was observed at 1000µg/ml concentration against 80% fractioned protein cuttlefish ink. The percentage of activity was highest in the 80% fractioned protein when compared to crude protein. Likewise Lu et al.,(1994) showed that cuttle fish ink could promote the phagocytic activity of macrophages and observed that there were significant differences in the phagocytic index inbetween the test and control groups. They also showed that the level of specific antibody against Meth A Sarcoma cell antigen in the serum from the mice treated with cuttle fish ink was significantly higher, indicated that cuttlefish ink could increase humoral immunity.
Lu et al., (1995) reported that cuttlefish ink elevated leukocyte quantity to improve hemopoiesis of marrow in mice.

He. S et al., (1999) studied the inducing effect of Sepia on the IL-2 level of mice. Xie et al., (2001) studied the inducing effect of sepia ink on the CSF (colony stimulating factor) level of mice. The results indicated that CSF stimulates the proliferation and differentiation of hemopoietic stem cell and many kinds of progenitor cells, increase the numbers of granulocyte, monocyte in blood and macrophage in tissue (Ganser et al., 1991; Guba et al., 1992). Wang et al., (2001) investigated the effects of Sepia on immunological function and they demonstrated that sepiia could induce the production of various cytokines and enhance the activity of immunocompetent cells. He. S et al., (2003) investigated the secretion of Interleukin-I in mice induced by sepia ink. They observed that ink can induce IL-I in BALB/c mice macrophage and showed the highest secretion was at 48 hrs. Pang et al., (2007) reported that the cuttle fish ink can promote the granulopoiesis in bone marrow and enhance the defensive system of organism

In the present investigation squid bone methanol extract showed 5% of activity at 10µg concentration and the activity was increased as the concentration was increased (10% at 100µg; 15% at 500µg) and reached the maximum of 16% at 1000µg concentration. The cuttlebone methanol extract showed minimum activity of 4% at 10µg concentration and followed by 6% at100µg, 14% at500µg and reached 15.5% at 1000µg concentration. Methanol fraction from squid bone powder have better scavenging activity, when compared to cuttle fish bone powder.

In the present study a protein fraction from squid ink and cuttle fish ink showed better macrophage scavenging activity compared to methanol extract from squid and cuttlefish bone powder and among the protein fraction, 30% fractionated squid ink showed
maximum scavenging activity. The normal rates of phagocyte activity of macrophages are compared with that of the phagocyte activity performed in the presence of sample. Increase in activity indicates the importance and better efficiency of samples in macrophage activity. So proteins can be targeted for cancer therapy.

Macrophages act indiscriminately against a broad spectrum of cancer cells. Macrophage directed immunotherapy of cancer is still in its infancy and the ability of tumoricidal macrophages to distinguish neoplastic from nonneoplastic cells presents an attractive possibility for treatment of the tumour cells which escape destruction by conventional treatments.
SCAVENGING ACTIVITY OF MACROPHAGE

Plate: 23

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B-blank  
PC-Positive Control  
CC-crude cuttle fish ink  
CS-crude squid ink  
80%C-80% fraction of cuttle fish  
30%S-30% fraction of squid ink  
CM-crude Methanol extract  
SM-squid Methanol extract

\[ \text{NBT reduction (\%)} = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD negative control}} \times 100 \]
Treatment conditions:

- 10 µg/ml
- 100 µg/ml
- 500 µg/ml
- 1000 µg/ml

% NBT reduction

Crude S. pharaonis Ink
Crude L. duvauceli Ink
Fig. 34

Treatment conditions

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Fig. 35

- Methanol fraction of *S. pharaonis*
- Methanol fraction of *L. duvauceli*