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Regulatory Action of *all trans* Cancer Acid on Metastasis Induced Lung Cell Metabolic Changes during Implantation of B16F10 Cancer Cells in C57BL6 Mice

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Abstract

The changes that occur during metastasis lodging is under intense research now to develop preventive new drugs to fight against the deadly metastasis. The molecular drug, *all trans* Retinoic Acid (ATRA) has regulatory effects on signal mediated metabolism. In this study, we have analyzed the metastasis facilitating metabolic changes in mice lung when a highly metastatic melanoma cell line (B16F10) having potency to lodge in lung was implanted via tail vein injection into C57BL/6 mice (1×10^6 cells/ml in PBS). One group of implanted mice were treated with 0.60 mg of ATRA per Kg body weight daily for 21 days. The alteration of protein, enzymatic and non-enzymatic antioxidants (SOD, Catalase, GPX, GSH) levels and the lipid profile with cholesterol level were evaluated in the lung tissues. The ATRA treatment caused 62.16% inhibition on metastatic nodule formation. Compared to normal mice, the cancer control mice showed an increased ($p\leq 0.01$) total protein, LPO and NO and a decreased antioxidant. In ATRA treated group, all these levels were reverted to near normal levels with a high significance ($p\leq 0.01$**) difference from untreated cancer mice. The lipid profile and cholesterol level also were altered in cancer and were normalized in ATRA treated group with high significance ($p\leq 0.01$**). All these results implies that the metabolic changes induced in the lung tissue during metastatic lodging of melanoma cells were prevented and regularized by the ATRA treatment *in vivo* which give a scope of anti-metastatic therapy using ATRA.**

Keywords: Lung metastasis, ATRA, C57BL/6 mice, melanoma cell line, Metabolism change

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INTRODUCTION

Cancer is a multi-factorial disorder, originating due to infection, inflammation, pollution-smoke, radiation, oxidative stress, diet, life style and genetics (Anand *et al*). Most of the lung cancer patients when diagnosed are at metastasis stage as it metastasizes very fast which may be due to rich blood flow. Hence, now the cancer researchers are concentrating more on management of cancer metastasis, fast progression as well as the associated changes in the body. The Vitamin A active metabolite, ATRA is a molecular therapeutic agent which has control over several genes involved in the molecular pathogenesis of cancer progression and metastasis (Cui et al) by binding to its receptor RARs and regulating target genes (Aranda *et al*.). ATRA has shown vital regulatory role in embryonic development of several tissues and organs (Romero *et al*). As an example, the functional retinoic acid response elements (RAREs) was found within flanking sequences of some of the most 3' Hox genes (Marshall *et al*.) which are the responder to ATRA during early embryonic development stages in control anterior/posterior patterning (Holland *et al*). ATRA alone or in combination is now used as a potent therapeutic agent for Acute Promyelocytic Leukaemia (APL) complete remission rate as high as 85% to 95% (Osman et al; Zhang et al and Chou *et al*) and was found to induce differentiation (Zuccari *et al*) and apoptosis (Altucci *et al*.). ATRA has been proven to treat vascular disorders and abnormal angiogenesis (Maiti *et al*.).

The oxidative stress and inflammatory mediators generated during cancer development aggravate the lung cancer progression and enhance the metastasis (Valavanidis *et al.*). The oxidative stress markers and inflammatory mediators set up a favourable tumour microenvironment at cancer initiation itself and enhance progression, promote angiogenesis, invasion, tumour cell migration, leading to metastasis. (Vaidya *et al*). Some studies have shown a relationship between retinoic acid and the generation of nitric oxide (NO) (Rafa et al., Grosjean *et al.*, Hirokawa *et al.*, Seguin *et al.*), prostaglandins (Mamidi et al.; Devaux *et al.,* Hill *et al.*) and the expression of cyclooxygenase 1 (COX-1) (Habib *et al*, Nusing *et al*) and COX-2 (Kanekura *et al*). Retinoid have various effects on cytokines by decreasing IL-12 and IFN as well as increasing IL-4 (Villarroya *et al*). Cell line implantation and metastasis is reported to generate several reactive species and radicals and the cancer condition itself reduces the antioxidant enzyme level in our body. However, it is unknown whether oxidative stress is the cause or the consequence of disease, but Reactive Oxygen Species (ROS) promotes tumor progression and make up the tumor microenvironment (Liou and Storz, 2010; Weinberg et al) which promote immune cell infiltration (Kotsafti et al). RoS activate cell survival signalling, leading to cancer cell migration and metastasis (Aggarwal et al.). However, reduction of these stress parameters and inflammation mediators help to manage the metastatic complications in lung cancer. Vitamin A and retinoid family have been traditionally recognized as chain breaking anti-oxidant or redox-active molecules (Khaper *et al*, Mantymaa *et* al). ATRA at very low level has enhanced the levels of MnSOD2 and Cu.ZnSOD1 to protect from oxidative damage in a neuronal cell line study (Ahlemeyer *et al.*). Lung metastasis involve oxidative stress and inflammation (Li *et al*.).

Lipids as the key membrane composition, source of energy and regulator of signal transduction pathways, plays vital role in cancer progression and undergo rapid change both at molecule and gene levels in cancer (Watkins *et al*). Study in mice has shown that the treatment with ATRA reduced body weight and adiposity independent of changes in food intake (Puigserver *et al.*) and has improved the glucose tolerance level (Felipe *et al*). ATRA has been proven to induce body fat loss which very well correlated with an activation of brown adipose tissue (BAT) (Bonet *et al.* and Puigserver *et al.*) and it was also reported to reduce adipogenic/lipogenic capabilities (Ribot *et al*.), thereby enhancing the oxidative metabolism and thermo genesis in white adipose tissue (WAT) depots, leading to fatty acid mobilization as well as healthy oxidation in other tissues (Mercader *et al*., 2006). Thus retinoic acid was found to regulate the adipose tissue development as well as deposition of lipid at different tissue**s** (Hiroshi *et al*.).

This study was therefore designed to analyze the vital changes happening in the lung of C57BL6 mice during cancer cell line implantation

and the regulatory role of ATRA in this condition by considering all these findings regarding metastasis associated metabolic and physiologic changes.

MATERIAL AND METHODS

Animals

Four to six weeks old mice (C57BL/6) were procured from the National Institute of Nutrition, Hyderabad, and subjected in this study. The mice were maintained in animal house at suitable condition as per the guidelines of CPCSEA during the experimental period by providing normal feed with water ad libitum.

Chemicals

ATRA was purchased from Sigma Chem. Co. (St. Louis, MO) and the other chemicals of analytical grade used were purchased from Himedia, India.

Cancer Cells

The melanoma cell line (B16F10) used for developing lung metastatic cancer in mice was obtained from the National Centre for Cell Science, Pune, India and sub-cultured in buffered medium (RPMI1640), supplemented with antibiotic, penicillin and streptomycin. They were recovered by harvesting with trypsin and EDTA and then (PBS, pH 7.4) wash was given.

Study Design and Groups

Four experimental groups (6 C57BL/6 mice per group) were included in this study. The group 1 mice was maintained as normal group and the mice of groups 2-4 were injected via tail vein with 0.1 ml of B16F10 melanoma cells (1×10^6 cells/ml in PBS) to develop metastatic mice model. The group 3 and 4 mice were simultaneously treated with i.p injection of 0.1 ml Olive oil and ATRA (0.60 mg per Kg body weight) in olive oil respectively for 21 consecutive days as per the literature (Suzuki et al.). Furthermore no mortality or behavioral changes were shown by the mice until 50 days of observation in acute toxicity study carried out earlier for the dose up to 1mg/Kg body weight. The protocol for these experiment was followed in line with the guidelines of CPCSEA and the approval for this study was obtained from the Institutional Animal Ethical Committee of Karunya University [IAEC/KU/BT/12/019]. On the 22nd day of metastasis induction and treatment, all the mice were sacrificed. The lung tissues were excised out, washed and observed visually through petri dish glass for metastatic nodules to count. From the number of nodules, we have calculated the percent of inhibition of nodule formation :

Inhibition Percent (%) = No. of nodules in metastasis control - No. of nodules in treated/ No. of nodules in metastasis control X 100

Then a small portion of it was cut, placed in a buffer formalin (10%), and embedded in paraffin for histologic studies. The remaining portion of lung tissues from each mouse were subjected to other analyses as detailed below.

Group 1:Normal mice

Group 2: B16F10 melanoma cell line implanted metastasis control mice

Group 3: B16F10 melanoma cell line implanted mice + Olive oil treatment

Group 4: B16F10 melanoma cell line implanted mice + ATRA treatment

Estimation of ATRA level in the tissue of lung

Lung tissues were homogenized with 10% ascorbic acid and ethanol. After vortexing, it was subjected to lipid extraction with n-hexane (2 ml), then vortexed. The hexane layer was collected after centrifugation for 10 minutes (2000 rpm) and air dried. The ATRA concentration in the residue after re-dissolving it in methanol (250µl) was then analysed using reversed phase HPLC system. The ATRA was detected at a wavelength of 310 nm with a mixture of acetonitrile in 4.5 to 6.5 ratio, run in the Phenomenex Luna column (250 mm×4.6 mm) at 1ml/minute flow rate. The peak area was calculated from the standard ATRA (400 to 2000ng/ml).

Total protein assay in lung tissues

A portion of lung tissue was homogeneized with buffer and the supernatant was separated by centrifugation. Then the total protein level was estimated against the standard protein bovine serum albumin (BSA) by the widely used standard method (Lowry *et al*.) and the optical density was measured at 680 nm. From the standard graph obtained from the increasing concentrations of standard BSA, the level of protein present in the lung tissue extract was calculated and expressed in µg/g tissue.

Evaluation of oxidative stress levels in lung tissue

The lung tissue was homogenised and the supernatant after centrifugation was subjected to analysis of various biochemical parameters which are related to oxidative stress induced in lung

during implantation of metastatic cell line. The assays for the following enzymatic antioxidants were performed using standard colorimetric methods described by the researchers in very early years as follows: The method described by Kakkar et al., was used for the assay of Super Oxide Dismutase (SOD)(Kakkar*et al*.). The Catalase assay was performed as describe previously (Sinha *et al*.). The Glutathione Peroxidase (GPX) assay was carried out by following the relevant literature (Rotruck *et al.*).

Similarly, the non-enzymatic antioxidant Glutathione (GSH) estimation was done in the supernatant of TCA (10%) precipitated homogenate by following the standard colorimetric method (Ellman *et al*.) and from the plotted standard graph and from which the GSH level in sample was calculated.

The extent of lipid peroxidation in lung tissue due to metastasis was assessed by measuring the production of thiobarbituric acid reactive substances (TBARS) by following the standard method (Ohkawa *et al.*) using MDA as standard. The assay for the generated nitric oxide (NO) was carried out in 250 µl of lung tissue homogenate by incubating it with Griess reagent for 15 minutes and reading at 546nm (John et al.)

Lipid profiling

The blood serum of experimental mice were subjected to lipid profiling along with estimation of total cholesterol level by following the standard Zak's method. Lipid profiling was done with the use of "Erba lipid profile kit. Briefly, the reagent was mixed with serum and the absorbance was read using colorimeter at 505 nm after 5 minutes incubation (37°C).

RESULTS

Metastatic nodules formed in lung

The number of nodules formed due to metastatic lodging in lung of all mice was visually counted and the mean number of nodules in each group of mice is given in Table 1. The effect of ATRA treatment on prevention was then calculated by comparing with the number of nodules found in the lung of metastasis control mice as follows and expressed as percent inhibition in Table 1.

The mean values given are from 6 mice and the p value is considered Non significant if it is > 0.05^{ns}; * means significant ($p \geq 0.05$), and ** means a highly significant ($p \geq 0.01$)

ATRA and total protein in lung tissue

The ATRA peak was obtained at RT value of 4.5 and from the standard calibration curve.

Table 1. Number of nodules found on lung and the percent inhibition by ATRA

Groups	Mean number of nodules	Inhibition (%)
1 - Normal	Nil	NA
2 - Metastasis control	59.52 ± 01	NA
3 – Metastasis + vehicle treated	56.61 ± 11 ^{ns}	4.88
4 – Metastasis + ATRA treated	22.52 ± 07**	62.16

NA – Not applicable

Table 2. The total protein level in lung tissue

Groups	Mean protein level (µg/g)	ATRA level (µg/ml homogenate)
1 - Normal	24.23 ± 12	0.223 ± 0.05
2 - Metastasis control	47.32 ± 10^{a**}	0
3 – Metastasis + vehicle treated	46.21 ± 17$^{a**\ b\ ns}$	0
4 – Metastasis + ATRA treated	31.22 ± 06 $^{a*,\ b**}$	0.476 ±.08 ^{a**}

NA – Not applicable

Normally the level of ATRA will be maintained in a lower quantity in serum and in lung. Hence in our study also the quantity of ATRA present in the lung of normal mice was found to be very low (0.223 ± 0.05) whereas, in the metastasis control group and the olive oil treated group the ATRA level was completely reduced and was undetectable. But the treatment with ATRA has shown an elevation ($p \geq 0.01$**) in the level of ATRA than the normal level in group 4 animal as shown in Table 2.

The increase in total protein level when compared to normal mice was found to be highly significant ($p \geq 0.01$**) in the metastasis control group as well as in olive oil treated group whereas the increase in ATRA treated group was minimum ($p \geq 0.05$*). ATRA treated group shown a greater difference from control group while a non-significance was observed in olive oil treated group as shown in Table 2. This may be resulted from the cell physiologic/functional changes due to increase in cell surface receptor, glycoproteins, lytic enzymes such as collagenase, cathepsin, plasminogen activator (cleave peptide bond) which favour mobility and dissemination of metastatic cells.

The values given are the mean± SD of 6 mice and the p value is considered Non significant if it is $> 0.05^{ns}$; significant if it is ≥ 0.05*, and highly significant if it is ≥ 0.01**. [a]Group 1 Normal Vs Groups 2-4; [b]Group 2- Control Vs Groups 3 and 4

Profile of Oxidative Stress Markers in the Lung Tissue

In this study we have observed a significant level of changes in lung antioxidant levels in the experimental groups during metastasis (Table 3). A highly significant ($p \geq 0.01$**) reduction in antioxidant enzymes was observed in metastasis control groups. Comparatively, the ATRA treated group has shown a highly significant increase in all these enzymes while, in the vehicle treated group, no significant reduction was observed in olive oil treated group (Table 3).

The mean values of 6 mice are given with SD.[ns] means not significant ($p > 0.05$); * means

Table 3. Levels of antioxidants in the lung tissue

Groups	Enzymatic SOD (U/Mg protein)	Enzymatic Catalase (µM/Mg protein)	Enzymatic GPX (µg/Mg protein)	Non-enzymatic GSH (µg/Mg protein)
1 - Normal	4.35± 0.02	0.091 ± 0.18	43.17 ± 0.14	1.93 ± 0.05
2 - Metastasis control	1.87± 0.03 [a**]	0.031 ± 0.02 [a**]	19.09 ± 0.08 [a**]	0.52 ± 0.16 [a**]
3 – Metastasis + vehicle treated	1.75± 0.06 [bns]	0.037 ± 0.04 [bns]	19.21 ± 0.13 [bns]	0.59 ± 0.13 [bns]
4 – Metastasis + ATRA treated	3.65± 0.11 [b**]	0.072 ± 0.05 [b**]	36.05 ± 0.07 [b**]	1.22 ± 0.06 [b**]

Table 4. Extent of lipid peroxidation and Nitric Oxide levels in the lung tissue

Groups	LPO activity (nM/g)	NO level (µM/g)
1 - Normal	12.21 ± 0.07	1.39± 0.07
2 - Metastasis control	40.33± 0.04 [a**]	3.98± 0.12 [a**]
3 – Metastasis + vehicle treated	40.52± 0.02 [bns]	3.65± 0.11 [bns]
4 – Metastasis + ATRA treated	20.14± 0.09 [b**]	2.04± 0.08 [b**]

Table 5. Lipid profile and cholesterol levels in the serum (mg/dl)

Groups	Cholesterol	TGL	HDL	LDL	VLDL
1 - Normal	110 ± 2	97 ± 4	42 ± 3	67 ± 1	21 ± 2
2 - Metastasis control	225 ± 1 [a**]	32 ± 2 [a**]	18 ± 2 [a**]	107 ± 2 [a**]	41 ± 1.5 [a**]
3 – Metastasis + vehicle treated	223 ± 2 [bns]	31 ± 3 [bns]	18 ± 3 [bns]	105 ± 1 [bns]	40 ± 3 [bns]
4 – Metastasis + ATRA treated	130 ± 1 [b**]	85 ± 1 [b**]	36 ± 1 [b**]	78 ± 3 [b**]	29 ± 2 [b**]

significant ($p \geq 0.05$) ** means highly significant ($p \geq 0.01$). Also, [a]Group 1 Normal Vs Groups 2 Control; [b]Group 2 Control Vs Groups 3 and 4 treatments

We have also observed a significant levels of changes in the extent of cell membrane lipid peroxidation as well as the reactive NO radical in the lung tissues among the groups of experimental mice during metastasis as shown in Table 4. The extent of lipid peroxidation and the NO level have increased in the metastasis control group in a highly significant ($p \geq 0.01$**) level while ATRA treated group has shown a highly significant ($p \geq 0.01$**) reduction than control. However, the olive oil treated group showed only non-significant difference from the control group.

The mean values from 6 mice are given with SD. [ns] means not significant ($p > 0.05$); * means significant ($p \geq 0.05$) ** means highly significant ($p \geq 0.01$).Here, [a]Group 1 Normal Vs Groups 2 Control; [b]Group 2 Control Vs Groups 3 and 4 treatments

Lipid Profile and Level of Cholesterol in the Lung Tissue

The results of lipid profile and cholesterol are given in Table 5. A highly significant ($p \geq 0.01$**) increase was shown in cholesterol and HDL levels in the metastasis control. On contrary to it, a highly significant ($p \geq 0.01$**) decrease was noticed for TGL in the control group. All these changes were brought to near normal level in the ATRA treated group while the olive oil treatment which showed similar alterations like control group could not make any reversion in the levels.

The data is given as mean values from 6 mice with SD. [ns] means not significant ($p > 0.05$); * means significant ($p \geq 0.05$) ** means highly significant ($p \geq 0.01$). Here, [a]Group 1 Normal Vs Groups 2 Control; [b]Group 2 Control Vs Groups 3 and 4 treatments

DISCUSSION

It is understood from the literature that most of the physiologic regulatory functions of Vitamin A mainly metabolism, cell growth, apoptosis and reproduction are exhibited through its one of the active metabolite, ATRA along with other retinoid (Mangelsdorf *et al.*). The chemotherapeutic role of ATRA as a cell differentiating molecular agent has become well known due to its efficient action on treating Acute Promyelocytic Leukemia (APL) and also by its key role in treating many other deadly diseases (Farooqui *et al.*). In addition to its therapeutic role ATRA has crucial role as chemo preventive agent against the development of cancer, angiogenesis and inflammation. In the past work which has been done to study the anti-inflammatory effect of ATRA in inflammatory arthritis has shown that the incidence of arthritis were lower in mice treated with ATRA than a normal mice (Yuji *et al.*). Some of the molecular actions of ATRA treatment on cancer were found to be arresting cell cycle at G1 as well as prolonging cell division rather killing tumor cells which is an identity for molecular therapy and also it could reduce DNA synthesis as well as colony formation. Being a differentiating agent it could also induce morphological changes in tumour cells (Wu *et al*.).

The cellular ATRA level is tightly regulated by two types of binding proteins such as CRABP I and II which direct it to nucleus for interaction with its high affinity receptors RARs (α, β, and γ) for exhibiting molecular action on target cells along with its isomer, 9-*cis*-retinoic acid which can also bind to RXRs (α, β, and γ) (Chen *et al.*; Mangelsdorf *et al*.; Heyman *et al*.). According to the variation in receptor types availability in each organ ATRA exhibits regulation of various cellular functions. Presently intense research is focused towards the therapeutic efficiency of ATRA for many solid cancers in addition to APL and vascular disorders (Zuccari *et al.*; Maiti *et al*.).

Recent studies have highlighted that the hypoxia condition in cancer cell infiltration induces ROS production and inflammatory responses which facilitate the tissue damage mediated migration of cancer cells, resulting in lung metastasis (Li et al.). A recent mice model study also has demonstrated that the hypoxia induced oxidative stress and inflammatory response promoted melanoma lung metastasis (Li et al.).In our study ATRA has shown an impressive antioxidant activity to reduce the free radicals present in the body by the different antioxidant enzymes. The previous reports states that the free radicals can damage the DNA and can cause a tumour cell proliferation. When ATRA was given to the cancer induced mice it has shown an impressive enzymatic antioxidant activity. Our study was done on SOD, Catalase, NO, GPx, GSH, LPO and the results states that ATRA has the

antioxidant property.

When these antioxidant levels are reduced in living systems, the excess free radicals generated during cancer metastasis and progression via activated LPO action as we have demonstrated in our study will facilitate further progression and establishment of cancer in metastasized site. It was reported by other studies also that the oxidative stress created by the free radicals have implication in the pathogenesis of many clinical disorders, including cancer (Narendirakannan *et al.*). They also have highlighted that there should be enough antioxidants or natural agents that are capable of augmenting the activity antioxidant enzymes to prevent implications of free radicals including cancer progression. In addition, the membrane lipids also plays key role in generating such free radicals as well as in signal transduction leading to alteration in cell growth and apoptosis (Watkins et al.).

On the basis of the previous reviews, our idea of doing the lipid profiling was to identify that when ATRA is given to the normal mice and the cancer cell line implanted mice what will be the effect on the lipid content of the body. There is no adverse change in the lipid content of the body for ATRA treatment however, there is a significant decrease in the cholesterol level in mice and increase in the LDL level. The result shows that ATRA has no adverse effect on the lipid content of the body and there were no significant side effect seen in ATRA treated mice.

On the basis of the result which came out of different parameter done to analyse the physiological effect of ATRA on lung cancer in mice has shown a potent effect of ATRA.

Since ATRA is already been use as a chemo-preventive drug for the APL, it can be used for the treatment of lung cancer also to prevent the metastasis and progression. Though ATRA is a lipid, by lipid profiling it is seen that it has not shown any adverse effect on the lipid content in mice body. The conclusion of all these parameters is that ATRA has potent anti-cancer drug properties against lung metastasis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors designed the experiments. SS & RP performed the experiments. VMBG analyzed the data and DDW wrote the manuscript along with BG. All authors read and approved the manuscript.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of NIH guidelines for animal use, Animal Care and Use Committee (NIH). The protocol for these experiment was followed in line with the guidelines of CPCSEA and the approval for this study was obtained from the Institutional Animal Ethical Committee of Karunya University [IAEC/KU/BT/12/019].

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