

Antiapoptotic Effect of Paclitaxel and *Ellagic Acid* against Mammary Cancer Induced by 7,12-Dimethyl Benz (a) Anthracene as Evaluated by Transmission Electron Microscopic Studies

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ABSTRACT In spite of great advances in cancer therapy, cancer remains the major cause of death throughout the world. The increasing resistance of cancer cells towards current anticancer drugs requires development of anticancer agents with a new mode of action. The ellagic acid had the most potent anticancer activity, also exhibit the flavonoid property and synergistic effect in combination with paclitaxel. The combinational chemotherapy regimen characterized by Transmission electron microscopic studies. The novel mechanism of action of paclitaxel is a antimicrotubular agent, its demonstrated single-agent activity, and its manageable toxicity profile make it an attractive candidate for inclusion of ellagic acid in combination chemotherapy regimens. Interaction of the two drugs may improve effectiveness in several ways. In addition to the therapeutic strategies already known, some recent reports indicate that new areas for the development of target selective drugs for the treatment of metastatic breast cancer were the simultaneous combination of two or more agents provided better results compare to the single dose.

INTRODUCTION

“Breast cancer is also a leading cause of cancer death in the less developed countries of the world. This is partly because a shift in lifestyles is causing an increase in incidence, and partly because clinical advances to combat the disease are not reaching women living in these regions,” says Dr. David Forman, Head of the IARC Section of Cancer Information, the group that compiles the global cancer data.

Breast cancer incidence is increasing and it is currently estimated that one in eight women develop the disease during lifetime. It is characterized pathologically by a proliferation of malignant epithelial cells within the mammary ductal-lobular system (Dickson and Clarks 1996). Generally, worldwide trends show that in developing countries going through rapid societal and economic changes, the shift towards lifestyles typical of industrialized countries leads to a rising burden of cancers associated with repro-

ductive, dietary, and hormonal risk factors. Incidence has been increasing in most regions of the world, but there are huge inequalities between rich and poor countries. Incidence rates remain highest in more developed regions, but mortality is relatively much higher in less developed countries due to a lack of early detection and access to treatment facilities. For example, in western Europe, breast cancer incidence has reached more than 90 new cases per 100 000 women annually, compared with 30 per 100 000 in eastern Africa. In contrast, breast cancer mortality rates in these two regions are almost identical, at about 15 per 100 000, which clearly points to a later diagnosis and much poorer survival. Breast cancer is the second leading cause of cancer deaths –about 16% in women after that of cancer of lungs (Greenlee et al. 2001). About 30% of the tumors develop in the breast during her 39 years of life and one in every 231 women faces this disease (Landis et al. 2001). In the United States more than 20,000 women are diagnosed with breast cancer every year, however very few risk prediction tests are known. Approximately 8% of American women can expect to be stricken with this disease some time during their life time (Moore et al. 1983).

Selective destruction of tumor cells without damaging normal cells is an important goal for

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cancer chemotherapy. The problem of cancer in the elderly will become increasingly more apparent. Plants, sources of phytochemicals with anti-cancer potential are reported to interfere with targets implicated in carcinogenesis and tumor cell biology makes them interesting tools in cancer research (Morse et al. 1993). The carcinogen used in the present study was Dimethyl Benz (a) anthracene. It has been reported to cause cancer in many cancer models like skin, mammary gland, oral, ovarian and salivary gland (Kim et al. 2003). Huggins et al. (1961), described the rat mammary tumor model which is also referred as a 'Huggins tumor model' and the histogenesis of the model has been studied by Russo (Russo et al. 1996, 2001). It is an indirect acting carcinogen that requires metabolic activation to yield an ultimate carcinogenic form (Moore et al. 2006).

Flavonoids may interfere in several of the steps that lead to the development of malignant tumors, including protecting DNA from oxidative damage, inhibiting carcinogen activation and activating carcinogen detoxifying systems (Ren et al. 2003; Birt et al. 2001). Researchers have become interested in flavonoids for their medicinal properties, especially their potential role in the prevention of cancers.

Ellagic acid is a nutraceutical and has very strong anti-oxidant properties. It is a potent anti-carcinogen and anti-mutagenic. It is also considered to be a cancer inhibitor which has the ability to restore apoptosis or normal cell death in cancer cells (Daniel et al. 2001). Paclitaxel is a novel antimicrotubule agent that promotes the assembly of microtubules from tubulin dimers and stabilizes microtubules by preventing depolymerization. This stability results in the inhibition of the normal dynamic reorganization of the microtubule network that is essential for vital interphase and mitotic cellular functions.

Apoptosis is genetically encoded cell death program consisting of morphological and biochemical changes (White et al. 1996; Williams et al. 1993). Cells undergoing apoptosis exhibit following characteristic features (1) mitochondrial depolarization, (2) membrane blebbing, (3) shrinkage of the nucleus, (4) condensation of chromatin, and (5) DNA degradation by endonucleases into fragments. Apoptosis is a tightly regulated process, which involves changes in

the expression of distinct sets of genes. Transmission Electron Microscopic studies evaluates the morphological alterations in the cell. The ultrasections viewed under this microscope gives us the idea of minute changes occurring in almost all organelles of the cell and is the most reliable technique. In the present study, the morphological alterations undergone after adding the drugs were evaluated which remained the backbone of the study.

MATERIAL AND METHODS

Animals

Aged Female Sprague Dawley rats weighing 150-200 g were purchased from National Institute of Nutrition, Hyderabad, India and were used throughout the study and they were maintained in the controlled environmental conditions of temperature and humidity on alternative 12 hr light/dark cycles. All the animals were fed standard pelleted diet (Gold Mohur rat feed, M/s. Hindustan Lever Ltd., Mumbai) and water *ad libitum*. Experimental animals were handled according to the university and institutional legislation, regulated by the committee for the purpose of control and supervision of experiments on Animals [CPCSEA, IAEC NO 02/068/06], Ministry of Social Justice and Empowerment, Government of India.

Experimental Protocol

The animals were divided into five groups of six animals each.

- Group I:* Control animals treated with Saline.
- Group II:* Dimethyl Benz (a) anthracene DMBA treated animals (20 mg/kg body wt., one injection by mammary Pad Air pouch technique).
- Group III:* Breast cancer bearing animals treated with Paclitaxel (33 mg/kg body wt., i.p) for 16 weeks (once in a week).
- Group IV:* Breast cancer bearing animals treated with Ellagic acid (100 mg/kg body wt., orally) for 16 weeks (twice in a week).
- Group V:* Breast cancer bearing animals treated with both paclitaxel and

Ellagic acid (as in Group III and Group IV) for 16 weeks .

Induction of Cancer

Production of Air Pouch

Air pouch was produced in the Sprague-Dawley rats by the method of Arun et al. (1984). About 2 ml of air was drawn in to the 5 ml syringe. It was autoclaved at 15 psi for 20 minutes in an airtight condition. The sterile air in the syringe was injected carefully just beneath the mammary fat pad subcutaneously so as to produce sterile air pouch. The air inside the pouch was allowed to stabilize for a day before administration of the carcinogen.

Administration of Carcinogen

20 mg of DMBA was weighed in a sterile vial and 0.5 ml of sterile saline and 0.5 ml of sunflower oil were added. The vial was stoppered and vortexed vigorously to obtain a uniformly dispersed emulsion. The single dose of DMBA was injected in to the air pouch. The growth of the tumor was measured at frequent intervals up to the 90th day when it attained maximum size. At the end of the experimental period, the animals were killed by cervical decapitation. The breast and liver tissues were used for further analyses

Transmission Electron Microscopy: The changes in the ultra structure of the breast tissue were studied by electron microscopy according to the method described (Moses et al. 2005). The cells of the sediment were re-suspended in phosphate buffered Karnovsky's fluid containing 3% glutaraldehyde and 2.5% paraformaldehyde (pH 7.4). Fixation was carried out for 2 hours at 4°C; after several washings in cold phosphate buffer (0.1 M, pH 7.4) the cells were postfixated for 1 hour in 1% phosphate-buffered osmium tetroxide, and the cell suspensions were finally centrifuged and placed in warm 1% agar solution. After solidification, the agar-included cell suspension was sliced by a razor blade. Blocks, not exceeding 1 mm³, were dehydrated in graded ethanols treated with propylene oxide and embedded in epoxy resin (Epon812); the blocks were then polymerized at 60°C for 36 hours. One-micrometer-thick sections

were cut with glass knives and stained with 1% toluidine blue in phosphate buffer. Thin sections (400-500 Å) of selected areas were stained with uranyl-acetate and lead citrate in accordance with Reynolds and observed with a Zeiss West Germany (Carl Zeiss 7082 Oberkochen, Germany) CEM902 electron microscope operating at 80 Kv.

RESULTS

Electron Microscopic Changes in Breast Tissue of Control and Experimental

Animals

Control: Plate (a) (10, 000x) and (b) (10, 000x) show normal cells lined by epithelial and endothelial cells.

DMBA Induced: Plate (c) (10, 000x) and (d) (15, 000x) show closely packed cells, the cytoplasm and nuclei vary in shape. The nuclei have irregular nuclear membrane. Irregular clumps of DNA are seen in the nucleus, a few dense granules appear and there is a degeneration of cells.

Paclitaxel Treated: Plate (e) (15, 000x) shows normal ultrastructural of nucleus with mild margination of chromatin.

Ellagic Acid Treated: Plate (f) (30, 000x) Show, the DNA appears to be dense and regeneration of cytoplasmic vacuolation is clearly seen.

Paclitaxel and Ellagic Acid Treated: Plate (g) (30, 000x) depicts, nuclear changes are seen which indicates apoptosis, the cells are shrunken and there is a clear fragmentation.

DISCUSSION

Transmission Electron Microscopic studies evaluates the morphological alterations in the cell. The ultrasections viewed under this microscope gives us the idea of minute changes occurring in almost all organelles of the cell and is the most reliable technique. In the present study, the morphological alterations undergone by the normal breast tissues, after the treatment with of DMBA, sole and combinational treatment of the drugs paclitaxel and ellagic acid were evaluated.

The researchers extended the potential application of the TEM studies to compare DMBA induced morphological changes between breast

cancer animals and normal animals. These data significantly suggests the differences in cancer progression in DMBA induced breast cancer bearing animals than the sole and combination treatment of the drugs ellagic acid and paclitaxel. Host factors including DNA repair capacity and carcinogen activation and/or detoxification may affect susceptibility to carcinogenic effects of DMBA. The breast cancer bearing animals shows closely packed cells, the cytoplasm and nuclei are vary in shape, nuclei have irregular nuclear membrane. Irregular clumps of DNA are seen in the nucleus, a few dense granules appear and there is a degeneration of cells.

In breast cancer bearing animals, the significant tumour progression may be due to the enormous proliferation of the cancer cells. The researchers have observed an induction of apoptosis following supplementation of paclitaxel along with ellagic acid to breast Cancer bearing animals. In drug treated animals, the cells were shrunk and there is a clear fragmentation which indicates apoptosis. Altogether, the nucleus shrinkage, clumping and alterations are seen signifying both early and late apoptosis. During apoptosis, certain characteristic morphological events such as nuclear condensation, nuclear fragmentation, and cell shrinkage, and biochemical events such as DNA fragmentation occur (Hsu et al. 1999; Shinoura et al. 1999). On drug treatment, the significant tumour regression may be due to the inhibitory action of the drug on tumour growth. Combinational treatment of the drugs ellagic acid and paclitaxel improve cell to cell communication which helps to prevent cancer cells from proliferation.

With respect to breast cancer in particular, the investigation of combination treatment of drug paclitaxel along with ellagic acid has generated much interest. Paclitaxel has repeatedly demonstrated an additive synergistic effect with other drugs. Paclitaxel thus hold the promise to have widespread applicability to cancer and other non-tumorigenic diseases. Clinical studies over the last decades have determined the most effective combinations of chemotherapeutic drugs. However, the combination treatments are associated with increased risk of myriads of side effects that may manifest several years after treatment (Mehren et al. 2005; Matesich et al. 2003). The best combination is yet to be achieved and clinical studies are kept on enduring. Devel-

opment of therapeutic strategies is, therefore, required for treatment without causing side effects. The strategy has been accomplished using medicinal plants, which have played a key role in the treatment of malignancy.

CONCLUSION

From the transmission electron microscopic studies in the experimental breast cancer, it is hoped that paclitaxel and ellagic acid can ultimately provide some avenues for the treatment of breast cancer in women and it can be considered in future for the evaluation of their clinical efficacy against breast cancer.

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