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Research Article

Scanning Electron Microscopy of Diosmin treated experimental animals on 7, 12-dimethylbenz (a) anthracene induced oral carcinogenesis in hamsters

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Abstract

On purpose of integrating the knowledge from basic researches with clinical studies, it can be stated that scanning electron microscopy is one of the most widely used methods nowadays to analyze the solid tumors. Most oral cancers are oral squamous cell carcinomas (OSCC) that arise from the epithelial lining of the oral mucosa. Given that the oral cavity is easily accessible, the disease lends itself to early detection; however most oral cancers are diagnosed at a late stage. The aim of the present study was to analyze morphological alterations in 7, 12-dimethylbenz (a) anthracene induced buccal tissues and Diosmin treated groups with scanning electron microscopy. Group I, served as an untreated control. Group II treated with DMBA alone (0.5%). Group III to Group V were painted with 0.5% DMBA + Diosmin (25, 50, 100mg/kgb.wt; dissolved in 0.5% DMSO). Group VI animals administrated with Diosmin alone (100 mg/kg b.wt) to exclude any toxic effects. We detected the presence of membrane ruffles and membrane blebs with lysed cells on DMBA alone treated buccal tissues and low membrane ruffles and blebs in Diosmin treated groups. Our results exhibit differential morphology in responses to changes in control, DMBA, DMBA+ Diosmin and Diosmin alone treated groups. The qualitative analysis of the scanning electron micrographs showed modified results in treatment with Diosmin (100 mg/kg b.wt). Our SEM images show membrane blebs on the cancer cell surface and lysed cells near the cancer cells. Membrane rufffles and lysed cells are characteristics indicative of apoptosis.

Keywords: Scanning electron microscopy, OSCC, DMBA, Diosmin, Membrane ruffles and Membrane blebs.

Introduction

Cells interact with different groups of macromolecules in the surrounding microenvironment that form the extracellular matrix (ECM). The ECM regulates cells advancement, Organization, function and signals from adjacent cells (Nikkhah *et al*., 2009). Cancer occurs when a normal cell fails to function properly, leading to abnormal cell growth that subsequently interrupts the Organization of tissue (Schindler *et al*., 2005). One of the distinctive properties of cancer cells is the presence of membrane ruffles on their surface. This was highlighted by Kaul-Ghanekar who used the tumor suppressor protein SMAR1 (Scaffold/Matrix Associated Region binding protein 1) as a phenotypic differentiation marker between cancerous and non-cancerous cells (Kaul-Ghanekar *et al*., 2009).

They demonstrated that untreated tumor cells exhibit a rough surface, whereas tumor cells treated with SMAR1-P44 peptide exhibit a smooth surface profile. They suggested that the rough surface of cancer cells may be a consequence of overall reorganization of cellular architecture as well as rearrangement of dynamic structures involved in cell motility and division (Kaul-Ghanekar *et al*., 2009).

The history of histology is closely related in its roots to the 19th Century discoveries regarding optical instruments. The end of the 19th Century and the beginning of the 20th century was also a period of great advances in histology with the use of new fixatives and stain combinations to produce histology

sections of excellent quality Fawcett (1966). The electron microscopy revealed a series of biological structures that could not be seen under the light microscope. Different methods for the specimen preparation were developed to explore the resolution power of this new equipment, which allowed the discovery of new organelles, the description of the macro cellular protoplasm components and the establishment of their functions. Since then, major promotion have been made regarding the morphological and functional aspects of cellular biology (Ham and Cormack, 1983). Nowadays, Scanning electron microscope (SEM) is largely used in research, and their images, when used in association, improve the understanding of the results.

In 1987 (Bird and Good) introduced a method to view the early development of experimental colon carcinogenesis in rodents. In whole-mount formalin-fixed colon preparations stained with methylene blue, they described how to detect small, protruded lesions by examining the entire mucosal surface under the light microscope. They described aberrant crypt foci (ACF) as putative preneoplastic lesions in the colon of carcinogen-treated rodents. Foci of early neoplastic changes at the mucosal surface observed by scanning electron microscopy (Barkla and Tutton, 1977). Although all the seputative preneoplastic lesions seem to be related, it is still not known whether any of them are real precursors of colonic tumors (Paulsen *et al*., 2005).

Squamous cell carcinoma

The histology of the squamous cells carcinoma is characterized by the proliferation of the cells of the stratum spinosum, which organize in cell groups, originating capillaries and nests, or individually, involving the conjunctive. The carcinoma can be graded as well or poorly differentiated. When observed individually, neoplastic cells are characterized by hyper pigmentation, pleomorphism, the increase in the number of mitotic figures and, sometimes, atypical mitosis (Neville and Day, 2002). Neoplasm of squamous cells usually present cellular heterogeneity, however, some parts of the tumor may present a more aggressive type of lesion.

Materials and Methods

Chemicals

7, 12-dimethylbenz (a) anthracene, Diosmin and analytical grade methanol, were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). All other chemicals used in this study were of highest analytical grade obtained from Sisco Research Laboratories and Himedia, Mumbai, India.

Animals

Male Syrian hamsters (80 ± 120 g), were obtained from National Institute of Nutrition, Hyderabad, India. Animals were housed in groups at constant temperature (23 ± 2˚C), and a light/dark (12 h/12 h) cycle. The animals were allowed to free access to food (VRK Nutritional Solutions, Maharashtra, India) and water (ad libitum) throughout the experimental period. The experiments were designed and conducted in accordance with the institutional ethical guidelines (Register number 160/1999/CPCSEA).

Experimental design

The male golden Syrian hamsters were divided into 6 groups of 6 each. Group I, served as an untreated control. The Group II treated with DMBA alone (0.5%) Group III to Group V were painted with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on the left buccal pouches using (No. 4 brush) to induce the buccal pouch carcinogenesis. Group III-V animals were orally administered with Diosmin (25, 50, 100mg/kgb.wt; dissolved in 0.5% DMSO) starting one week before the exposure to the carcinogen and continuing on alternate days of the DMBA painting until the animals were sacrificed. However, Group VI animals were orally administrated with Diosmin alone (100 mg/kg b.wt) to exclude any toxic effects.

Sample collections

At the end of the experimental period, the hamsters were sacrificed by cervical decapitation under anesthetic conditions (Xylazine 30 mg/kg, i.p.). The control and experimental animal buccal tissues were immediately removed, washed using ice-cold phosphate buffer solution (pH 7.4), and then the buccal tissues were used for assessment of SEM analysis. Scanning electron microscopy was carried out in Department of Manufacturing Engineering, Annamalai University, using a JSM 6610 LV scanning electron microscope (Jeol, England Ltd.) operated at 15 KeV.

Results

Scanning electron microscopy analysis was carried out to investigate the histological interactions between tumor tissues and the Diosmin treated buccal tissues. All the groups were made to undergo standard scanning electron microscopy procedures. Ultra structural analysis revealed distinctive features of buccal cancer and its relationship with the Diosmin treated groups. Fig.1a shows normal surface images with no membrane ruffles and lysed cells. Membrane ruffles were found to exhibit 'cabbage-like' structures due to the cancer cell outer membrane peeling off. Furthermore, lysed cells were detected near the cancer cells, indicating the occurrence of apoptosis (Fig.1b). This demonstrates the presence of membrane ruffles on cancer cells at a higher magnification. To enhance the survival rate of cancer cells, displacement of the prominent leader cancer cell advance followed by other cancer cells looking shrunken was observed. Apoptosis was indicated by membrane blebs scattered on the cancer cell surface, contributing to the invasive properties of cancer cells by engulfing of normal cells (Fig. 1.b). It is thought that these abnormalities have a direct influence on the invasiveness of cancer cells. When coming to the Diosmin treated groups Fig.1c&d (25 and 50mg/kg.b.wt.) illustrates the mild membrane ruffles and blebs with decreased lysed cells. Our study shows 100mg/kg.b.wt recovered the demolished membrane ruffles and blebs which looks somewhat similar to control group (Fig.1a). Finally the Diosmin alone treated group (Fig.1f) shows no formation of membrane ruffles and cell lysis.

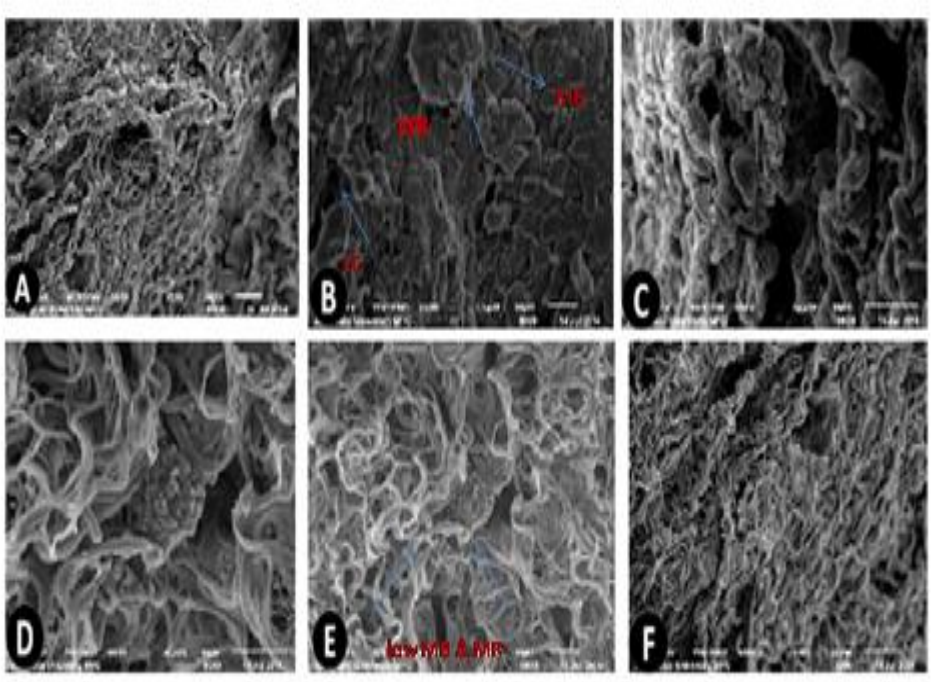

Fig.1. Scanning electron microscopy of the treated rats. A) Untreated control animals B) DMBA (0.5%) alone group. MR = Membrane ruffles; MB = Membrane blebs; LC = Lysed cells. C) DMBA + Diosmin (25mg/kgb.wt). D) DMBA + Diosmin (50mg/kgb.wt). E) DMBA + Diosmin (100mg

Discussion

The understanding of the morphology and microcirculation plays an important role in understanding the phenomena that occur in different microenvironments of tumors (Fukumura *et al*., 2010). A major role played by tissue microenvironments is to maintain normal cell behavior. Thus, any changes induced by Oncogenes or other external influences which cause rearrangement of the cytoskeleton will transform the properties of cells. Cytoskeleton rearrangement leads to Disorganisation of the membrane of cancer cells. This is a result of changes to internal biopolymer protein, an important component of the cell cytoskeleton, which subsequently alter the surface of cancer cells

(Brinkley *et al*., 1980). We observed surface morphology of classic elevated ACF in this late stage of carcinogenesis was, in principle, the same as we had previously reported for the early stages in rats (Paulsen *et al*., 1994), in mice (Paulsen *et al*., 2001) and in hamsters (Paulsen *et al*., 1996). Interestingly, these small, elevated lesions had been observed with scanning electron microscopy in DMH- or AOM-induced rat mucosa (Cook *et al*., 1984). Our SEM images demonstrate the presence of membrane ruffles on the buccal cancer cells. Several authors have speculated on the nature and role of membrane ruffles. Johnston *et al*. and Stoica *et al*. reported that membrane ruffles are specialized plasma membrane ultra structures which contain fine actin filaments. Furthermore, they suggested that membrane ruffles have a role in the growth, development and motility of cancer cells, and so are important in determining the metastatic potential of cells (Johnston *et al*., 1995).

Investigation of the role of membrane ruffling in cell behavior using a breast cancer cell line (Rajah *et al*., 2001) Their findings led them to conclude that the presence of membrane ruffles on the surface of cancer cells facilitates ligand-receptor binding processes by acting as a platform for adhesion foci and signal transducing events that lead to the activation or suppression of genes. Furthermore, they proposed that membrane ruffling is one of the cancer cell's properties. In their study, they showed that conditioned media from NIH 3T3 fibroblasts (3T3-CM) used as a chemo attractant in matrigel invasion chambers increases the invasiveness of breast cancer cells. Additionally, they found that MCF-7 breast cancer cells treated with 3T3-CM exhibit an increased rate of membrane ruffling of the plasma membrane and concluded that 3T3-CM increases the invasiveness of breast cancer in direct correlation with increased membrane ruffling (Rajah *et al*., 2001). Our SEM images reveal that the presence of membrane ruffles on the buccal tumor cancer cells gives them a rougher appearance than normal breast cells. Similar finding has previously been reported by (Kaul-Ghanekar *et al*., 2009) who found that control tumor samples (mice injected with melanoma cells) show surface roughness, whereas treated tumor samples (mice injected with melanoma cells followed by the tumor suppressor protein SMAR1-P44) exhibit a smooth surface (Kaul-Ghanekar *et al*., 2009). Our SEM images show membrane blebs on the cancer cell surface and lysed cells near the cancer cells. Membrane rufffles and lysed cells are characteristics indicative of apoptosis.

Conclusion

It is possible to relate the images captured by SEM, pointing out areas that suggest a link between the morphology of the Diosmin treated and tumor tissues of SEM analysis provides a new dimension to the detection of cell surface abnormalities. Our data suggest that the presence of membrane ruffles and lysed cells could act as benchmarks for further diagnosis regarding stages of Oral cancer. Further research should be focused on the role of invasion-assisting proteases, which is at the edges of cellular protrusions**.**

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