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Full Length Article

Effect of celecoxib and cisplatin combination on apoptosis and cell proliferation in a mouse model of chemically-induced colonic aberrant crypt foci

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The use of cisplatin for the treatment of cancer is accompanied by dose-dependent adverse effects. In colorectal cancer, there is upregulation of cyclooxygenase-2 (COX-2) expression increases prostaglandin E2 (PGE2) which in turn depresses apoptosis and potentiates invasion, angiogenesis, cell-proliferation and metastasis. This study investigates a possible synergistic function for celecoxib in cisplatin-based chemotherapy against chemically-induced colon carcinogenesis in mice. Mice received fifteen injections of 1,2-dimethylhydrazine dihydrochloride (DMH; 20 mg/kg/week), s.c. to induce colon carcinogenesis and the normal control group received equal volumes of normal saline. Mice were randomly divided into five groups, (I) normal control group, (II) DMH control group (III) DMH + cisplatin (4 mg/kg/week, i.p.) group, (IV) DMH + celecoxib (10 mg/kg/day by gavage) group and (V) DMH + cisplatin + celecoxib group. Drugs were administered starting from week eleven until the end of the experiment (week 15). Colon specimens were used to evaluate histological grades, examine intratumoral expression of Bcl2, BAX and caspase-3 and the number of proliferating nuclei. The combination of cisplatin and celecoxib was effective against malignant transformation in mice with DMH-induced colonic aberrant crypt foci (ACF). The combination group showed improvement in histological grading, the highest caspase-3 expression and the lowest Bcl2/BAX ratio and reduction by approximately 50% of immunoreactivity for proliferating cell nuclear antigen (PCNA) compared to DMH control group. Addition of celecoxib to cisplatin regimen promotes apoptosis, suppresses tumor proliferation and augments the antitumor effect of cisplatin chemotherapy in the mouse model of DMH-induced ACF.
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1. Introduction

Colorectal carcinoma is a major contributor to cancer burden worldwide. It is also considered as a leading cause of cancer-related mortality [1]. In Egypt, the Middle East Cancer Consortium previously reported lower rates of colorectal carcinoma ($6.9/10^5$ for males and $5.1/10^5$ for females) during the period of 1999–2001 [2]. However, an increased incidence of colorectal carcinoma was observed among young patients under the age of 40. This increased trend is alarming as it is usually associated with poor prognosis [3–5].

Colorectal carcinogenesis includes changes in the colonic mucosa both at the histological and the molecular levels [6]. Morphologically, aberrant crypts can be observed as a single altered crypt or as a cluster of altered crypts that form a focus termed; aberrant crypt foci (ACF). Histologically, ACF are heterogeneous group of intraepithelial lesions that exhibit variable features, ranging from almost normal or mild atypia to severe dysplasia. Indeed, ACF represent preneoplastic lesions that indicate early stages of colorectal carcinogenesis in both rodents and humans [7,8]. ACF are characterized by greater size, increased pericryptic zone and thicker epithelial lining [9]. Hence, examining the development of ACF is valuable in rodent chemoprevention studies

Cyclooxygenases have distinct expression pattern and biological activity. In colorectal carcinoma, cyclooxygenase-1 (COX-1) level remains unaltered but cyclooxygenase-2 (COX-2) expression is up-regulated [10–12]. COX-2 produces prostaglandin E2 (PGE2), which enhances the resistance to apoptosis and the potential for invasion, angiogenesis, cell-proliferation as well as metastasis [13]. Further, double knockout mice model of adenomatous

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polyposis coli and the COX-2 genes showed lower number and reduced intestinal polyp size [14]. Furthermore, COX-2 is reported to be over-expressed in a variety of solid tumors such as prostate, colon, lung and liver cancer. Therefore, drugs that inhibit COX-2 enzyme can be promising chemo preventive agents.

Cisplatin, cis-diammine dichloroplatinum (II) Pt $(NH_3)_2(Cl)_2$ is a platinum-containing chemotherapeutic drug which is used for the treatment of several types of cancers, lymphomas or germ cell tumors [15]. The chloride atoms on cisplatin are displaced by water molecules which enroll the platinum atom to preferentially bind to guanine nucleobase initiating DNA cross linking and subsequently triggering apoptosis [16]. On the other hand, celecoxib is a non-steroidal anti-inflammatory drug with higher selectivity toward inhibition of COX-2 enzyme. Steinbach et al., demonstrated that celecoxib mitigates polyps formation in patients with familial adenomatous polyposis [17]. Celecoxib exerts its anti-tumorigenic effects through inducing apoptosis in tumor cells via the activation of the anti-apoptotic kinase [18]. Interestingly, celecoxib produces antitumor effect in COX-2-deficient tumors in nude mice model and promotes apoptosis in cells not expressing COX-2 [19]. Therefore, the action of celecoxib against cancer does not solely depend on inhibiting COX-2 action.

The present study aimed to examine the chemopreventive effect of cisplatin and celecoxib combination in 1,2-dimethylhydrazine (DMH)-induced colon ACF in mice. We evaluated the expression of intratumoral caspase-3, Bcl2 and BAX to investigate the intrinsic apoptotic pathway. Furthermore, we used the proliferating cell nuclear antigen (PCNA) immunostaining to examine the impact of the combination therapy on cell proliferation.

2. Methods

2.1. Drugs and chemicals

Cisplatin (Unistin vials, 10 mg/10 ml solution) was obtained from Eimc United Pharmaceuticals (Cairo, Egypt) and was diluted with sterile saline. Celecoxib was a gift from Amoun Pharmaceutical Company (El-Obour City, Egypt). Celecoxib was suspended in 2% sodium carboxy methyl cellulose (Na-CMC; ADWIC, Cairo, Egypt) solution.

2.2. Preparation of dimethylhydrazine

1,2-Dimethylhydrazine dihydrochloride (Sigma-Aldrich®, MO, USA) was diluted with EDTA-normal saline (1 mM/L) and the pH was adjusted to 6.5 with sodium hydroxide.

2.3. Animals

Male Swiss mice (20–26 g) were purchased from the Egyptian Organization for Biological Products and Vaccines (Cairo, Egypt). Mice were maintained in a 12 h dark/light cycle at 25 ± 3 °C and. Mice were housed in groups of six in well-ventilated clean plastic cages and all possible efforts were done to limit suffering of the mice. Cage substrate was replaced each day with food and tap water *ad libitum*. Experimental procedures were approved by the institutional research ethics committee at Faculty of Pharmacy, Suez Canal University. Date of approval: June 2014.

2.4. Experimental design

After a 1-week acclimatization period, sixty male mice were randomly divided to five experimental groups; each group consisted of 12 animals. Group **1** (Normal control group) in which the mice received normal saline (20 ml/kg/week, sc). Group **2** (DMH control group): Mice injected with DMH (20 mg/kg; sc) weekly for a total of 15 injections [20]. Group **3** (DMH + cisplatin): mice received weekly injections of DMH and given cisplatin (4 mg/kg/week, i.p.) during the last 5 weeks of the experiment. Group **4** (DMH + celecoxib group): mice received weekly injections of DMH and administered celecoxib (10 mg/kg/day) by gavage during the last 5 weeks of the experiment. Group **5** (DMH + cisplatin + celecoxib group): mice received weekly injections of DMH and received cisplatin (4 mg/kg/week, i.p.) plus celecoxib (10 mg/kg/day) [drugs were given from week 11 to week 15]. Mice were monitored in a daily manner to detect any type of discomfort and to register the number of surviving mice.

2.5. Tissue processing

At the end of week 15, mice were anesthetized with ether and sacrificed by cervical dislocation. Then, mice were autopsied and the colon was excised, opened along the horizontal axis and flushed using ice-cold saline. Next, colon sections were flattened and fixed in 10% phosphate buffered formalin for 1 day. Tissue samples were processed, embedded in paraffin wax, cut into sections (4-μm) and finally stained with hematoxylin and eosin (H&E) for histological diagnosis of tumors [21].

2.6. Histopathological examination of colon specimens

Colon sections were examined under a light microscope (Olympus CX21, Japan). A low power (×10 magnification) was used to visualize the whole tissue sections while scoring was performed using a high power (×40 magnification). Morphometric scoring was employed using image analyzer. A grading system was established for the degree of cell dysplasia. In which dysplasia was considered as aberrant structure, hyperplasia was considered upon observing increasing size and number of cells, mucosal inflammation was considered when inflammatory cell infiltration, focal inflammatory cell aggregation, lymphoid proliferation, congested blood vessels and fibrosis were detected. Score 0 was assigned for normal sections that appear free from any signs of dysplasia, hyperplasia or inflammation. Score 1 was assigned when no dysplasia but mild inflammatory reaction was observed. Score 2 was assigned for moderate inflammatory reaction with or without dysplasia. Score 3 was assigned for dysplasia/or hyperplasia with severe inflammation. Score 4 was assigned in cases of severe inflammatory reaction with dysplastic or hyperplastic activity and fibrosis [22].

2.7. Immunohistochemical staining

Immunohistochemical staining was performed employing a 3-step indirect technique based on the labelled avidin-biotin peroxidase complex (ABC) method. Colon sections were deparaffinized, rehydrated and boiled with 0.01 M citrate buffer, pH = 6.0, for 15 min for antigen retrieval. Next, tissue specimens were maintained at room temperature and covered for 1 h in 1% bovine serum albumin. This was followed by an overnight incubation (at 4 °C) with the different primary antibodies. On the next day, biotinylated secondary antibodies were added to the tissue sections. Then, the avidin-biotin-peroxidase complex was added (LAB-SA detection system; Invitrogen) Visualization of the reaction was performed using a 0.05% solution of 3,3-diaminobenzidine which produces a dark brown precipitate. Tissue sections were subjected to counterstaining with Mayer's hematoxylin, and dehydrated in ascending alcohol series. Then, sections were cleared in xylene and finally mounted with DPX. Sections for the negative control were incubated without the primary antibodies. In this study, rabbit polyclonal antibodies against caspase-3 (1:100), mouse monoclonal antibodies against Bcl2 and BAX (1:100,

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