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Chemotherapy Induces Oral Mucositis in Mice Without Additional Noxious Stimuli[1]

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Abstract

Oral mucositis (OM) is a serious side effect of cancer chemotherapy. The pathobiology of oral mucositis remains incompletely understood due to lack of appropriate models which recapitulate the human condition. Existing rodent models are intraperitoneal and require radiation, chemical or mechanical injury to the chemotherapy protocol to induce oral lesions. We aimed to develop an OM mouse model that is induced solely by chemotherapy and reproduces macroscopic, histopathologic and inflammatory characteristics of the human condition. Female C57BL/6 mice were given intravenous 5-Fluorouracil (5-FU) injections every 48 hours, for 2 weeks. A high daily dose of intraperitoneal administration was tested for comparison. Mice were monitored daily for weight loss. Epithelial histomorphometric analyses in tongue, esophageal and intestinal tissues were conducted coupled with assessment of apoptosis, cell proliferation, neutrophilic infiltration and the integrity of adherens junctions by immunohistochemistry. Neutropenia was assessed in peripheral blood and bone marrow. Tissues were analyzed for pro-inflammatory cytokines at the protein and mRNA levels. Daily intraperitoneal administration of 5-FU led to rapid weight loss and intestinal mucositis, but no oral inflammatory changes. Intravenous administration triggered atrophy of the oral and esophageal epithelium accompanied by reduction in cell proliferation and increased apoptosis. Coincidental with these changes were up-regulation of NF-κB, TNFα, IL-1β, GM-CSF, IL-6 and KC. Despite neutropenia, increased oral neutrophilic infiltration and reduced E-cadherin was observed in oroesophageal mucosae. We developed a novel experimental tool for future mechanistic studies on the pathogenesis of chemotherapy-induced OM.

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Introduction

Mucositis is an inflammatory condition resulting from damage to the oroesophageal, gastrointestinal and genitourinary tract mucosae, following cytotoxic cancer therapies [1]. Its severity and duration varies with the dose and the type of drug used, but in certain cases it can lead to compromised nutrition and chemotherapy dose reduction or postponement due to severe pain or diarrhea. In fact, mucositis can be of such severity that patient survival is adversely impacted [2]. Oral mucositis affects 40–80% of patients undergoing chemotherapy and even though it can be subtle at first, it gradually becomes more severe after 7 to 10 days of treatment [3].

One of the most commonly used drugs associated with mucositis is 5-fluorouracil (5-FU). 5-FU inhibits thymidylate synthase and depletes intracellular thymidine triphosphate pools [4] arresting cells in S phase [5]. 5-FU has also been proposed to interfere with the activity of ribosomal RNA binding protein, at the level of pre-ribosomal RNA processing [6]. The exact mechanisms of inflammation are not fully understood, yet some progress has been made over the last several years with the development of rat, hamster [7] and mouse [8–11] mucositis models. Current intraperitoneal (IP) mouse models are either based on a single high dose of 5-FU (100–500 mg/kg), or a smaller daily dose (30–50 mg/kg) [10–12] for up to 4 weeks. IP rodent protocols are technically simple and trigger

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intestinal mucositis reproducibly but do not significantly affect the oral mucosa, unless head and neck irradiation, [13] local mechanical or chemical injury [7,14] are included.

In murine IP models the intestinal mucosa is exquisitely more sensitive to chemotherapy than the upper alimentary tract mucosa, thus animals lose intestinal function rapidly and have to be euthanized prior to developing oral lesions [9–12]. Increased susceptibility of the intestinal mucosa may be related to proximity of the drug or the type of epithelium (simple columnar versus stratified squamous epithelium) and different epithelial turnover rate, which is about 1–4 days in small intestinal epithelium and 14–21 days in buccal mucosa and esophagus. [15] Turnover rate differences can affect both the sensitivity and rate of recovery from mucositis, but are not sufficient to explain differences in the inflammatory response to chemotherapy.

Due to lack of a clinically relevant chemotherapy-induced oral mucositis animal model the cellular and molecular events involved in its pathogenesis remain incompletely understood. The aim of this study was to compare a high dose IP 5-FU chemotherapy model to a low dose, intermittent, intravenous mode of administration of longer duration, resembling human anti-cancer regimens. We focus our investigation on the effects of 5-FU on the oral mucosa with the main goal of developing a reliable model of oral mucosal inflammation in response to chemotherapy. For the first time we also analyze the bone marrow immunosuppressive effect of 5-FU and its effect on the structural integrity of the mucosal barrier, which may have a bearing on the increased susceptibility to opportunistic infections. Finally, to begin to understand the mechanism involved in the site-specific responses to chemotherapy we perform a comparative analysis of the oral, esophageal and intestinal histopathologic and inflammatory responses.

Materials and Methods

Animals

Six to twelve-week-old female C57BL/6 mice were used (Jackson Laboratories, animal protocol 100,965–1017). Animals were housed with access to pelleted food and water ad libitum in a temperature-controlled environment with a 12-hour light/dark cycle. Mice were monitored daily for signs of morbidity and body weights were recorded every 24-48 h.

5-FU Administration

Mice received 100 mg/kg 5-FU (Sigma, St. Louis, MO, USA), daily for 4 days, via IP injection and were sacrificed on day 5. Alternatively, mice received intravenously (IV, via tail vein) 50 mg/kg 5-FU every 48 hours, from day 1 to day 13 and euthanized on day 14 by carbon dioxide exposure. This dose and frequency of administration was chosen as it is effective in substantially reducing tumor size in a xenograft mouse model [16], and is within the therapeutic intermittent dose range in humans [17,18]. Control groups received PBS, either IP or IV. Tongue, esophagus, jejunum, femurs and tibiae were retrieved at necropsy. Animal experiments were repeated thrice, unless otherwise stated.

Macroscopic and Histopathologic Examination

Tongues were stained with 1% toluidine blue in 10% acetic acid for 1 minute, followed by repeated washes with acetic acid, to reveal surface erosive or ulcerative lesions [19]. The percentage of toluidine blue positive surface area (excluding excision trauma) was calculated using the Image J software. Tissues were then fixed in 4% (v/v) paraformaldehyde solution in PBS for 2 hours at 4 °C, and processed for paraffin or OCT embedding. Epithelial thickness in tongues and esophagi, and villus length in the jejunum were measured in H&E stained tissues using Image J. Three mice per group, with 3 fields per sample and 5 measurements per field, were analyzed, in a blinded fashion. Images were obtained using a Zeiss Axio Imager M1 microscope and an EC-Plan-Neofluar 920-NA 0.5 air-objective and using the AXIOVISION-SE64 Rel. 4.9.1 program.

Determination of Cell Proliferation and Apoptosis in Mucosal Tissues

Mitotically active oroesophageal and intestinal crypt cells were detected using a Ki67-rabbit pAb (Cell Signaling technology, Danvers, MA) as described previously [20]. Cell apoptosis was evaluated using the DeadEnd™ Colorimetric TUNEL® System, according to manufacturer's instructions (Promega, Madison, WI).

RNA Extraction and Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Mouse tongues and jejunum were homogenized using a POLYTRON homogenizer, and the supernatants were beat by zirconia beads (Ambion, Waltham, MA) with phenol:chloroform: isoamyl alcohol (25:24:1 v/v/v, Thermo Fisher Scientific, Waltham, MA). RNA was purified using the QIAgen RNeasy® Mini Kit and concentrations/quality were determined using a NanoDrop device (Thermo Scientific, Waltham, MA). Complementary DNA was synthesized with SuperScriptIII Cells Direct® cDNA Synthesis kits (Invitrogen, Carlsbad, CA). Reverse-transcription quantitative polymerase chain reaction was performed with a Bio-Rad CFX96 cycler and the iQ® SYBR Green Supermix (Bio-Rad, Hercules, CA). Primer sets for each gene are shown in supplemental Table 1 (supplemental). Fold changes were calculated using the $\Delta\Delta^{CT}$ method.

Laser Capture Microdissection (LCM)

Tongues were snap-frozen in dry ice-cooled 2-methylbutane (Acros, Geel, Belgium), and embedded in cryomatrix compound (Thermo Fisher Scientific, Waltham, MA). A Leica CM 3050S (Leica Microsystems, GmbH, Nussloch, Germany) cryostat with installed

Table 1. Primer sets for each gene used to amplify the selected pro-Inflammatory cytokines.

	Forward	Reverse
NF-kB	5′-ATGGCAGACGATGATCCC TAC-3′	5′-TGTTGACAGTGGTATTTCTGGTG -3′
IL-1β	5′-GACACTGTTCCTGAACTCAAC T-3′	5′-ATCTTTTGGGGTCCGTCAACT-3′
TNF-α	5′-GGAACACGTCGTGGGATAATG-3′	5′-GGCAGACTTTGGATGCTTCTT-3′
GM-CSF	5′-GGCCTTGGAAGCATGTAGAGG-3′	5′-GGAGAACTCGTTAGAGACGACTT-3′
IL-6	5′-GACAAAGCCAGAGTCCTTCAGAGA G-3′	5′-CTAGGTTTGCCGAGTAGATCTC-3′
KC	5′- GCCAATGAGCTGCGCTGTCAGTGC-3′	5′-CTTGGGGACACCTTTTAGCATCTT-3′
GAPDH	5′-TCCTACCCCCAATGTGTCC-3′	5′-CTCTTGCTCAGTGTCCTTGCT-3′