Experimental and Toxicologic Pathology 66 (2014) 235–242

Contents lists available at ScienceDirect

Experimental and Toxicologic Pathology

journal homepage: www.elsevier.de/etp

Characterization of azoxymethane-induced colon tumor metastasis to lung in a mouse model relevant to human sporadic colorectal cancer and evaluation of grape seed extract efficacy

Molly M. Derry[a], Komal Raina[a], Rajesh Agarwal[a,b], Chapla Agarwal[a,b,*]

[a] Department of Pharmaceutical Sciences, Skaggs School of Pharmacy, University of Colorado Anschutz Medical Campus, Aurora, CO, United States
[b] University of Colorado Cancer Center, University of Colorado Anschutz Medical Campus, Aurora, CO, United States

ARTICLE INFO

Article history:
Received 28 August 2013
Accepted 22 February 2014

Keywords:
Colorectal cancer
Lung metastasis
Biomarkers
Chemoprevention
Grape seed extract

ABSTRACT

The second leading cause of cancer-related deaths (both genders combined) in the United States is colorectal cancer (CRC). This emphasizes the need to develop both effective therapies for CRC patients and pre-clinical models mimicking human disease that carry translational potential in drug-development. Notably, at present there are no *in situ* models of CRC metastasis to lung. In our azoxymethane-induced colon tumorigenesis study in A/J mice assessing grape seed extract (GSE) efficacy, during necropsy we also found multiple lung nodules suggestive of colon tumor metastasis to lung that were significantly inhibited in GSE fed group. Both histopathological and molecular studies were performed to characterize and establish the origin of these lesions in lung. Histologically these nodules were determined as adenocarcinoma of mucin origin. Molecular analyses by immunohistochemistry (IHC) and RT-PCR revealed strong protein and transcript levels of colon specific markers CDX2 and CK20 in these lung nodules compared to uninvolved control lung tissue. Vis-à-vis, these nodules also showed minimally expressed lung specific biomarkers, specifically surfactant D and TTF-1, in IHC analysis. Additionally, 0.25% GSE supplementation in diet (w/w) decreased the incidence of these lung nodules by 53% and their total number by 66%. Together, the characterization of this unique *in situ* mouse model of CRC metastasis to lung provides translational opportunities in developing effective therapies to clinically manage and treat CRC at the advanced stage. Moreover, GSE efficacy in inhibiting CRC metastasis to lung in this model further supports its translational potential in controlling CRC growth, progression and metastasis in patients.

1. Introduction

In most industrialized countries, cancer is the major cause of disease-related deaths; the majority of these deaths are a result of cancer metastatic growth (Taketo, 2011). Furthermore, colorectal cancer (CRC) is the second leading cause of cancer-related deaths (both genders combined) in the United States, and is expected to result in 50,830 deaths during 2013 alone (http://seer.cancer.gov/csr); most (~75%) of these CRC cases develop sporadically (Migliore et al., 2011). At the time of diagnosis, clinical and pathological staging of CRC is closely related to survival rate. For example, according to the United States Department of Health & Human Services, the overall 5-year relative survival rate of CRC confined to the bowel wall is 90% (http://seer.cancer.gov/csr), and a further progression of CRC to the lymph nodes decreases the 5-year survival rate to 70% (http://seer.cancer.gov/csr). If distant metastasis occurs, the survival rate decreases to 12.5% (http://seer.cancer.gov/csr); common organ sites of clinical CRC metastasis, include liver, lung, peritoneum and ovaries (Van Loon and Venook, 2012). The tissue specific markers have been important tools utilized by diagnostic pathologists to determine metastatic tumor origin. For example, caudal type homeobox 2 (CDX2) is a highly sensitive marker of primary and metastatic colorectal adenocarcinomas, exhibiting 100% accuracy of intestinal tumors (Werling et al., 2003). Similarly, the biomarkers such as thyroid transcription factor-1 (TTF-1) are specifically expressed with in the lung, thyroid, and brain tissues (Boggaram, 2009).

Traditional treatment for CRC includes adjuvant chemotherapeutic agents and surgery, depending on the location of metastatic growth; however, the current chemotherapeutic agents induce toxicity, which also includes the risk of death (Ohe, 2002). Moreover,

* Corresponding author at: Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, 12850 E. Montview Blvd., C238, Room V20-2118, Aurora, CO 80045, United States. Tel.: +1 303 724 4057; fax: +1 303 724 7266.
E-mail address: Chapla.Agarwal@UCDenver.edu (C. Agarwal).

http://dx.doi.org/10.1016/j.etp.2014.02.003
0940-2993/

current chemotherapeutic agents are not capable of increasing long-term patient survival or possess the ability to cure metastatic disease. With such limitations in mind, it is evident that there is a clinical need to develop non-toxic, effective, long-term treatment regimens to control CRC growth, progression and metastasis to distal organs. In this regard, an additional approach to the current chemotherapeutic agents would include natural products that have limited or no toxicity and could be administered for long-term to prevent, suppress or reverse colon carcinogenesis (Rajamanickam and Agarwal, 2008). Correspondingly, there are multiple reports indicating that one-third of all cancer deaths in the US could be prevented through high consumption of fruits and vegetables or their bioactive components (Kaur et al., 2009; Kelloff et al., 1996; Rajamanickam and Agarwal, 2008). Natural agents such as grape seed extract (GSE) have been shown to be bioavailable, safe and efficacious during clinical trials against multiple cancer types; additionally GSE exhibits significant efficacy against CRC in pre-clinical mouse models (Eich et al., 2012; Kaur et al., 2009; Velmurugan et al., 2010a,b).

Scientific progression in the field of cancer molecular biology and genetics has fostered the development of effective patient therapies, which are attributed to the development of pre-clinical animal models that mimic the human disease condition. However, the pre-clinical research field has not yet developed an efficient and effective model of carcinogen-induced colon tumorigenesis where colon tumors metastasize to lung; notably, this CRC metastatic tumor formation in lung accounts for 10–20% of the diagnosed patient population (Van Loon and Venook, 2012). Additionally, this lung metastasis patient population might not have the option of surgery, and thus, further decreasing the probability of patient survival. With this in mind, the aim of this study was to characterize lung nodules found in A/J mice following azoxymethane (AOM) exposure and establish their origin as colon tumors. Rodent experimental models of colon carcinogenesis have been utilized for over 80 years; the use of rodent models has a number of advantages to study the pathogenesis of CRC, including model reproducibility, access to different genetic backgrounds, and that these models recapitulate human cancer development (Rosenberg et al., 2009). To mimic human sporadic CRC development, there are a variety of carcinogens that induce colon tumors; however, AOM-induced colon carcinogenesis takes the advantage of the organotropism of the carcinogen where AOM specifically induces tumors in the colon and small intestine with highest incidence in the distal region of the colon (Rosenberg et al., 2009). Notably, AOM-induced colon tumorigenesis model is the most commonly used pre-clinical animal model of human sporadic CRC (Rosenberg et al., 2009); however, previous studies have not reported the metastasis of colon tumors to the lung in this model. Additionally, investigating GSE efficacy in an *in situ* pre-clinical animal model of CRC metastasis is highly desirable, which combined with the vast pre-clinical GSE efficacy studies in various models of colon carcinogenesis, would help provide much needed evidence to initiate clinical trials investigating GSE efficacy against human CRC growth, progression and metastasis to distal organs including lung.

2. Material and methods

2.1. Reagents

GSE-standardized preparation was a gift from Kikkoman Corp. (Nado City, Japan). The preparation composition is as follows: 89.3% procyanidins, 6.6% monomeric flavanols, 2.24% moisture content, 1.06% of protein, and 0.8% of ash, as reported recently (Derry et al., 2013; Velmurugan et al., 2010a,b). Purchased antibodies include anti-CDX2, anti-CK20, anti-Surfactant D and anti-TTF-1 (all from Abcam). Anti-mouse and anti-rabbit horseradish peroxidase (HRP) secondary antibodies were from Invitrogen (Carlsblad, CA) and Cell Signaling Technology (Beverly, MA). RNA was isolated *via* the Qiagen RNeasy Kit, amplified *via* the Qiagen RT^2 RNA qPCR kit. Additionally, RNA transcript was quantified *via* specific RNA TaqMan primers for *Cdx2* from Life technologies (Grand Island, NY) and primers for mouse *Ck20* from Invitrogen.

2.2. Animals and treatments

Male A/J mice were purchased from Jackson Laboratory, and experiments were done with an approved protocol by IACUC (Derry et al., 2013). AOM was purchased from Sigma (St. Louis, MO) and dissolved in saline. GSE was mixed in AIN-76A powder diet at 0.25% (w/w). Animals, maintained under standard conditions with free access to water and food (AIN-76A powder diet), were divided in to 3 groups, and treated as: (1) untreated control group ($n = 20$), (2) AOM (positive) group ($n = 35$), injected with 5 mg/kg dose of AOM i.p. once a week for 6 weeks, and (3) AOM + 0.25% GSE ($n = 35$), GSE containing diet feeding started 2 weeks post last AOM injection and continued till end of the study (28 weeks). Body weight and diet consumption were recorded weekly. At 43 weeks of age mice were sacrificed, entire lung was excised, gently flushed with ice-cold PBS, gross lung nodules counted and fixed flat in formalin, and were either embedded in paraffin for pathological and immunohistochemical (IHC) studies or frozen in liquid nitrogen for protein isolation, or stored in Qiagen RNA*later* (Valencia, CA). Additionally, colon and small intestine tumors were also documented and stored as published recently (Derry et al., 2013).

2.3. Pathological and immunohistochemical (IHC) analyses

Fishers' Exact test was used to compare incidence/number/type of pathological lesions in different groups. The significance level was set at $P \leq 0.05$ for all tests. Paraffin-embedded sections (5 μm) of lung tissue including nodules were subjected to standard H&E staining as well as IHC analysis as described previously (Derry et al., 2013; Velmurugan et al., 2010a). Primary anti-body dilution in each case was 1:100. Negative staining controls were used for each protein. Microscopic analyses were performed using Zeiss Axioscope 2 microscope; photomicrographs captured by Carl Zeiss AxioCam MrC5 camera with Axiovision Rel 4.5 software. All IHC data are shown as mean ± standard error mean (SEM) and are representative of 10 fields/section of 4 different mice per group. Statistically significant difference between untreated control, AOM and AOM + GSE groups was analyzed using one-way ANOVA (Sigma Stat 2.03, Jandel Scientific, San Rafael, CA). P values of ≤0.05 were considered significant.

2.4. RNA isolation and real time-RT-PCR (qRT-PCR)

Total RNA was isolated (from 20 mg tissue in each case) employing Qiagen RNeasy Kit, as per vendor' protocol, and RNA concentration was determined with a NanoDrop 2000 (Thermo Scientific). Next, Qiagen RT^2 RNA qPCR kit was used following manufactures protocol and the First Strand cDNA Synthesis Reaction was stored at −20 °C. In qRT-PCR, the commercially available and pre-validated TaqMan primer/probe set used was: *Cdx2* (*CDX2; Mm01212280_m1*). The primer set for *Ck20* was designed 5′-GCGTTTATGGGGGTGCTGGAG-3′ (F) and 5′-AAGGCTTGGGCGGTGCGTCTC-3′ (R). mRNA levels of triplicate samples from each group were measured by real-time quantitative reverse transcription-PCR using ABI PRISM 7700 at the Molecular Biology Shared Resources of the University of Colorado Cancer Center. Quantities of specific mRNA in each sample were normalized to