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Hepatocellular carcinomas are promoted by tocopheryl acetate but eliminated by tocopheryl succinate

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HIGHLIGHTS

- An alarming increase in hepatocellular carcinoma development was found to result by.
- An increased level of dietary tocopheryl acetate in the presence of aflatoxin B1.
- Tocopheryl succinate, in contrast to the dietary acetate ester, caused a marked decrease in the level of carcinomas.
- Poor diets caused an increase in the base level of carcinomas.
- Non-soybean protein reduced the carcinoma rate in a vitamin/mineral deficient diet.

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ABSTRACT

Background: The major causes of hepatocellular carcinomas are Aflatoxin, hepatitis B and hepatitis C viruses.

Alpha tocopherol and its acetate and succinate esters have each been reported as counteracting cancer development in humans and rodents. We have investigated their salutary effect in both poor and high quality diets in rainbow trout *Oncorhynchus mykiss* as a model.

Methods: Hepatocellular carcinomas (HCCs) were induced in rainbow trout by dietary aflatoxin B1 (AfB1). A matrix of different levels of several vitamins and vitamin analogues were included in selected diets as possible anticancer agents. Identification of HCCs was made by histopathology.

Results: 1.) **Elevated dietary tocopheryl acetate (E-Ac) caused a marked increase in liver size and in AfB1-induced HCCs in rainbow trout.** 2.) Poor diets increased the HCC incidence. 3.) Elevated dietary tocopheryl succinate (E-Su) nearly eliminated HCC development in fish fed complete diets. Tocopheryl succinate in poor diets reduced HCCs by 77% compared to tocopheryl acetate diets. 4.) Trans-retinoic acid also reduced HCC incidence. 5.) Vitamins A and D deficiency caused tumor increases but had no effect on liver size. 6.) The use of casein and dextrin in the place of soybean textured vegetable protein, in poor diets nearly eliminated the HCC risk. 7.) Trout sera showed all three vitamin forms; free α-tocopherol (E-OH), tocopheryl acetate (E-Ac) and tocopheryl succinate (E-Su), from diets containing any of these vitamin analogues, suggesting both de-esterification and trans-esterification. 8.) E-Su is discussed in the light of an anti-cancer agent that is non toxic to normal tissue but that cohorts to it are needed.

Conclusions: Increased dietary E-Ac escalated AfB1 induced HCCs and caused hepatomegaly in rainbow trout, while E-Su eliminated the HCC risk as shown by histopathology.

1. Introduction

Liver cancer is estimated to be the 6th most common type of cancer in the world with 749,700 new cases in 2008. Between 75% and 90% of these in humans are hepatocellular carcinomas (HCCs) [1]. It is estimated to be the 3rd leading cause of cancer deaths in the world and the 9th leading cause in the United States [2]. The American Cancer Society estimates that in 2015 in the United States there would be about 35,660 new cases and 24,550 deaths due to liver cancer. http://www.cancer.org/cancer/livercancer/detailedguide/liver-cancer-what-is-key-statistics (accessed 16 June 2015).

Hepatitis B virus (HBV), hepatitis C virus (HCV) and dietary aflatoxin B1 (AfB1) are the most significant contributors to the risk for HCCs [3] and [4]. Modest levels of AfB1 increased the risk of HCCs by

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30 fold in HBV infected Korean men [5].

A recent cluster involved AfB1 contaminated pet food that killed as many as 76 dogs in southeast United States [6] (http://recalledpetfoodsettlement.com).

Aflatoxins (several isomers) are the result of fungi (Aspergillus species) growth on feedstuffs and are found in many types of improperly stored feed including peanuts, pistachio nuts, milk, corn, soybeans and other grains. The aflatoxin limit for pistachio nuts is 20 part per billion (ppb) [7]. The US food Safety Regulations include a limit of 20 ppb for total aflatoxins (B_1, B_2, G_1 and G_2) in all foods except milk which has a limit of 0.5 ppb for aflatoxin M_1. Higher limits apply in animal feeds [8].

Enhancing resistance to tumor development as well as removing the causative agents are both desirable. Vitamin E is one of several vitamins that are both credited and discounted as being effective in preventing various types of cancer. Vitamin E in one study had no affect on breast cancer rate in postmenopausal women but no indication was given as to the form of the vitamin assessed [9]. Induced adenocarcinoma incidence in rats was reported to not be significantly altered by tocopheryl acetate (E-Ac) dietary increase [10]. Dietary elevation of dl-alpha tocopheryl acetate (the synthetic racemic mixture of the vitamin) in diets (1.8 gm/Kg diet) in double transgenic mice genetically altered for transforming growth factor α(TGF-α)/c-*myc*, caused a reversal of hyperplasia, and eliminated 65% of the adenomas and 100% of the HCCs but, in contrast, a marked reduction in apoptosis [11].

In contrast to the variable effects of tocopheryl acetate (E-Ac), the results with tocopheryl succinate (E-Su), have been consistently beneficial. E-Su while protecting normal cells [12] caused apoptosis in human breast cancer cells [13], human prostate cancer cells [14], murine leukemia cells [12] and rat colon pre-cancer crypts [15] and was synergistic with cellular apoptosis factors and radiation treatment [16,17,18], and [19].

Reactive oxygen species (ROS) such as the hydroxide radical ($OH^{\cdot}$) have been implicated as causative agents in carcinogenesis against which vitamin E acts as an ROS scavenger to protect against cancer. However the vitamin can only act in the scavenger role when it exists as the free alcohol but not as the acetate nor succinate ester [20] and [21].

We sought to test the protection given against HCC incidence by vitamins A, C, E and Vitamin A metabolites cis- and trans-retinoic acids. Rainbow trout were used as a model for combating cancer in mammals because this species is known to be susceptible to HCC induction by dietary aflatoxin B1 [22]. Our results confirm that the intact vitamin E ester forms of both acetate and succinate are retained partially in the blood and that trans-esterification of both acetate and succinate on tocopherol occurs in rainbow trout suggesting an equilibrium exists between E-Su, E-Ac and the free tocopheryl (E-OH) (See Appendix C). Some studies have addressed possible differences in activity of the different analogues of the vitamin as between **α**-tocopherol versus **β**, γ or **δ** form and especially between the acetate and the succinate ester of **α**-tocopherol [11] and [23].

This study reports the *in vivo* prevention, in rainbow trout livers, of AfB1 induced HCCs by the use of E-Su. In contrast, increased dietary E-Ac caused a several fold increase in trout HCC incidence and up to a three-fold increase in liver size.

2. Methods and materials

Rainbow trout were obtained both as eggs and as swim-ups from Nisqually Trout Farms Inc. in Lacey, WA. Two weeks after swim-up they were all fed the same base diet[19] for a period until they were over 1 g in size when they were then put on the respective test diets shown in Appendix A. All fish tanks were 28 L and received 1 L per minute, 10° C Spring water to maintain 9 ppm oxygen. The complete diet feed was made into a gel from the respective pure ingredients purchased principally from Sigma chemical Co. The compromised diets substituted food grade textured soybean protein (TVP), wheat flour and powdered milk in the place of casein, dextrin and alpha-cell. Aflatoxin was dissolved with propylene-glycol-methyl-ether acetate and chloroform to produce the required concentration then added to the oil mixture of each diet. The completed diet slurries were cooled then frozen at −34° C till they were used, but fresh diets were made about every month or six weeks.

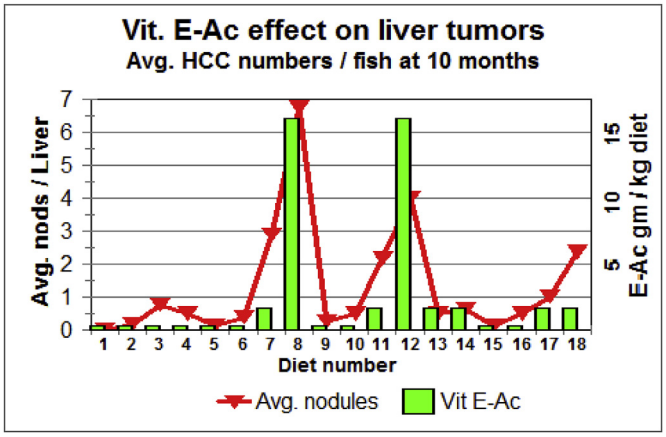

Fig. 1. The average number of carcinoma nodules per liver slide as a function of vitamin E-acetate in each of 18 different diets. With each level of vitamin E, three other vitamins were tested, A, C and retinoic acid.

Fish serum was collected after centrifugation into vials, frozen and shipped frozen on ice for analysis of free Vitamin E and its esters at the Michigan State University Animal Health Diagnostic Laboratory (AHDL) in East Lansing MI and at Exova Inc. in Portland, Oregon. Starting at 9 through 16 months on test diets, fish were sampled from each diet group at the same length of time on the diets. There was more than one time length of test growth period for most of the experiments. For sampling, the fish were killed with 3 aminobenzoic acid ethyl ester (MS222) at the rate of 200 mg/L water.

The livers were excised intact, weighed, photographed, the visual tumor rank recorded and were then put into 10% neutral buffered formalin for later HCC histopathology and tumor identification by RW (CellNetix Pathology and Laboratories) in Olympia, WA. The histopathology results were reported as follows 1). Was there any carcinoma (data not shown), and 2). The number of tumor nodules in each liver slide. Histopathological interpretation was blindsided as to treatment and gross appearance of the livers. Gross nodule appearance was consistent with carcinomas histologically. Histology examples of the HCCs are shown in slide photos following the text references. Following that are Figs. 7–11of examples of the whole livers from the respective diets.

Appendix A following the photos has the base ingredients for all diets used.

The conditions under which the fish were reared and cared for followed the "Guide for the care and Use of Laboratory animals: Eighth Edition 2010" National Research Council; ISBN: 0-309-15401-4, (2010), Pages 77–78 - Aquatic Animals. This is beyond the United States Animal Welfare Act of 1966 which does not apply to cold blooded animals.

(see: http://animalresearch.thehastingscenter.org/u-s-law-and-animal-research/).

3. Statistical analysis

Appendix B has a summary of the statistical probability of the variance of HCCs on test diets versus the control diet groups using the Students *t*-test of results shown for data used in Figs. 1–6.

4. Results

4.1. Results: histopathological identification of cellular carcinomas

Tumor characteristics include enlarged cell size, an expansile nodule, an absence of cytoplasmic lipid droplets, inflammatory cells from