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ORIGINAL ARTICLE

The role of ascorbic acid, selenium, and glutathione on benzo[a]pyrene-induced carcinogenesis in wistar rats

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Summary

Purpose: *The carcinogenic action of polycyclic aromatic hydrocarbons (PAHs) can be inhibited by endogenous or exogenous compounds. This study was designed to elucidate the modifying action of 3 endogenous inhibitors- ascorbic acid (vit C) used alone, and selenium (Se) used in combination with glutathione (GSH).*

Materials and methods: *Chemical carcinogenesis was induced by benzo[a]pyrene(BaP). A hundred wistar rats were divided into 3 groups: the first group (G I) consisted of 42 animals, representing the control group. The two experimental groups (G II and G III) consisted of 38 and 20 rats, respectively. All groups were injected with BaP(10.08 mg subcutaneously-s.c). The first experimental G II was given only vit C (520 mg in 2% sugar solution per os - p.o.). The second experimental G III was given Se (0.1 mg p.o.) with GSH (200 mg p.o.). Tumor incidence and mean survival time were determined. Histological examination of the developed and excised tumors took place following death. The carcinogenic potency (CP) and anticarcinogenic potency (AP) of the substances used were calculated.*

Results: *A statistically significant difference regarding the mean survival time in the two experimental groups (238.4±31 days and 344.9±48 days, respectively) compared to the control group (183.8±28 days) was found ($p < 0.001$). The CP of each of the 3 groups was 54.3, 41.2, and 28.9 units, respectively. The AP of vit C used alone was 13.1 units, representing a significant anticarcinogenic effect. The combination of Se + GSH showed an AP of 25.4 units, resulting in a significant prolongation of the mean survival time, which is considered a potent anticarcinogenic effect. Furthermore, a statistically significant difference was found also when the mean survival time of G III animals was compared with G II.*

Conclusion: *Vit C on its own and Se in combination with GSH represent strong endogenous inhibitors that can inhibit/reduce the carcinogenic action of BaP-induced carcinogenesis in wistar rats. The combination therapy used offered better in vivo results.*

Key words: ascorbic acid, benzo[a]pyrene, carcinogenesis, glutathione, selenium, wistar rats

Introduction

Among PAHs, those possessing oncogenic properties are composed principally of 4, 5 or 6 aromatic rings. The oncogenic activity is related to their chemical structure which provokes a high pi-electron density at definite molecular positions, thus increasing their reactivity and fixation with the cell components. Regions with a raised pi-electron density are called k-regions and represent the carcinophore groups of the oncogenic PAHs [1]. Substituents which decrease the electronic charge of the k-region also decrease the carcinogenic power, and substituents which increase the electronic charge usually increase the carcinogenic power.

Benzo[a]pyrene (3, 4-benzo[a]pyrene; BaP), a carcinogenic PAH derivative, is a potent carcinogenic

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agent with strong oncogenic activity. Inhibitors of PAH-induced carcinogenesis embrace endogenous (or physiological) and exogenous (or synthetic) anticarcinogenic substances. These compounds when administered to carcinogen-susceptible laboratory animals effectively postpone or completely protect them from the oncogenic action of PAHs. Wistar rats represent an excellent living model for the examination of the anticarcinogenic effect of physiological and synthetic substances which could act as inhibitors of the PAH carcinogenesis. The local development of a tumor in the area of application of the BaP in wistar rats approaches 100% under certain experimental conditions [2,3]. Furthermore, wistar rats need a relatively short time for tumor induction (palpable tumor in 40-70 days). Wistar rats die of progressing malignant tumors 150 to 250 days after the BaP injection. Normal lifespan of wistar rats is between 800-900 days. Tumors caused by BaP are not histologically uniform and show characteristic tissue differentiations, as follows: polymorphic squamous cell carcinomas, mixed carcinosarcomas, as well as leukemias, lymphomas, mammary adenocarcinomas, gastric adenocarcinomas, mesotheliomas, and cancer of the bladder.

Vit C is a good inhibitor of hyalouronidase, the enzyme that liquefies and breaks down tissues. Vit C is an endogenous inhibitor of carcinogenesis and acts as an antioxidant [4,5]. Linus Pauling, Ewan Cameron and their co-workers performed systematic studies on terminal human cancer cases using vit C as a supportive treatment with good results [6]. The trace metal Se belongs to the group of potent antioxidants, which are strong inhibitors of PAH-induced carcinogenesis [4,7,8]. The anticarcinogenic action of some newly synthesized metal complexes (Pt, Sn) has also been investigated with good results [3,9-12]. GSH, carrying the functional group -SH characteristic of thiols, is an endogenous inhibitor of PAH-induced carcinogenesis, having stronger antitumor activity than other thiols, e.g. L-cysteine. Organic sulphur compounds also promote cancer cell division. In general, thiol sulphydryl groups are involved in cell division. If these are blocked, no cell division occurs. Thus, a number of compounds that react with sulphydryl-containing cell constituents are actually excreted by the organism as mercapturates and are known inhibitors of the PAHs carcinogenic action in rat skin cancers. GSH maintains an optimum cellular redox potential with its chemical depletion or intracellular redistribution being associated with the onset of apoptosis [13].

In the present work the inhibition/reduction of BaP-induced chemical carcinogenesis using vit C alone and the combination of Se with GSH was evaluated.

Materials and methods

In this study, 100 male wistar rats genetically similar and living under the same laboratory conditions were used. The animals aged from 60-80 weeks and weighed 200 g to 250 g on starting the experiment. A single dose of 10.08 mg BaP (Merck) was dissolved in 1ml tricaprylin (Sigma) shortly before s.c. injection in the upper right shoulder of each rat. This BaP amount induces tumors in nearly 100% of the rats. The animals were previously anaesthetized using ether alcohol.

Wistar rats were divided in 3 groups as follows:

Group I (G I): Control group; 42 wistar rats injected with BaP alone.

Group II (G II): 38 wistar rats injected with BaP and and given 520 mg vit C in 2% sugar solution daily p.o. until death. Animals do not like the acidic taste of ascorbic acid solutions; they prefer sweet drinks which have the added effect of making the animal more thirsty, thus, assuring the daily consumption of all the ascorbic acid dose administered. Furthermore, complementary calories are provided to the animals. Calories contained in the sugar solution are beneficial, mainly in cases of cancer cachexia.

Group III (G III): 20 wistar rats injected with BaP. Se 0.1 mg and GSH 200 mg daily p.o. were administered until death. Selenium was used in the form of sodium selenite pentahydrate (Merck, Darmstadt); GSH was provided by Santen. We have shown in previous studies that the combination of the anticarcinogens enhances the antineoplastic activity; therefore in G III we used these two agents in combination.

After animals' death, a detailed autopsy took place. Their body weight was measured and the tumor mass was excised and weighed. A histological examination followed. The animals' survival time (in days) from the time of BaP injection, as well as the tumor incidence found in autopsy after the animals' death, were determined more accurately than the average tumor induction period.

The experimental results were evaluated and compared using the formula [2,3,5,12]:

$$\frac{\text{tumor incidence (\%)}}{\text{survival time (in days)}} \times 100$$

to indicate the CP of BaP. Similarly, the AP of the substances used to reduce the CP was also calculated (degree of inhibition.) According to the above formula we calculated:

$$CP_{BaP} = \frac{\text{tumor incidence (\%)}}{\text{survival time (in days)}} \times 100$$

The AP of the substances used was determined by calculating the difference between the CP of BaP when administered alone, with that when BaP was administered simultaneously with anticarcinogen(s).

Hence, if:

$CP_{BaP} = A$

$CP_{BaP+vitC} = B$

then,

$AP_{vitC} = A\text{-}B = CP_{BaP}\text{-}CP_{BaP+\ vitC}$

Similarly, if:

$CP_{BaP+Se+GSH} = C$

then,

$AP_{Se+GSH} = A\text{-}C = CP_{BaP}\text{-}CP_{BaP+Se+GSH}$

The results taken were statistically evaluated by the Student's *t*-test.

Results

In G I, the tumor induction was 100% whilst the mean survival time was 183.8±28 days. One animal in G II developed a cyst at the site of BaP injection instead of a tumor mass and lived 820 days. Thus, the tumor induction in G II was 97.4% and the mean survival time was 238.4±31 days. In G III these parameters were 100% and 344.9±48 days, respectively. According to Table 1 data and referring to the aforementioned formulas, we calculated:

$$C_{BaP} = \frac{100}{183.8} \times 100 = 54.3 = A \text{ (G I)}$$

$$CP_{BaP+vitC} = \frac{97.4}{236.4} \times 100 = 41.2 = B \text{ (G II)}$$

hence,

$$AP_{vitC} = A\text{-}B = 54.3\text{-}41.2 = 13.1$$

Similarly,

$$AP_{Se+GSH} = \frac{100}{344.9} \times 100 = 28.9 = C \text{ (G III)}$$

hence,

$$AP_{Se+GSH} = A\text{-}C = 54.3\text{-}28.9 = 25.4$$

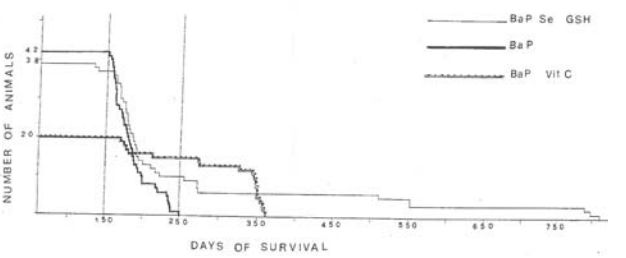

Figure 1. Death rate curve of the 3 studied groups

Statistical evaluation of the survival rate between groups G I and G II, G I and G III, as well as between G II and G III is outlined in Table 1. A statistically significant difference ($p < 0.001$) was found between G I and G II, between G I and G III, as well as between G II and G III. Tumors removed ranged in weight from 40g to 150g. The most common histological type of the tumors developed was fibrosarcoma. Figure 1 shows the death rate curve of wistar rats in the 3 groups studied.

Discussion

Numerous articles have been published dealing with possible mechanisms of PAHs carcinogenesis. Emphasis was given to some important physicochemical and biological properties of the carcinogens and their interaction with vital cell constituents. The carcinogenic activity of aromatic compounds in relation to their molecular geometry was first reported by Berenblum followed by Arcos and Argus who studied the two-stage mechanism of carcinogenesis [14,15]. PAHs carcinogens have been shown to interact with nucleic acids. To explain chemical carcinogenesis, Miller proposed a general theory that has a significant impact on cancer research [16]. Some of these suggestions are *i)* most chemical carcinogens that are not chemically reactive must be converted metabolically into a chemically reactive form *ii)* the activated metabolite is an electronic reagent *iii)* this activated metabolite reacts with nucleophilic groups in cellular macromolecules to initiate carcinogenesis.

Table 1. Tumor incidence, treatment, mean survival time, carcinogenic and anticarcinogenic potency, and p-value in the 3 groups studied

Group	*No. of animals*	*Tumor incidence (%)*	*Treatment*	*Mean survival time (days)*	*CP*	*AP*	*p-value (<0.001)*
G I	42	100	BaP	183.8±28	54.3		I *versus* II
G II	38	97.4	BaP+vit C	238.4±31	41.2	13.1	I *versus* III
G III	20	100	BaP+Se+GSH	344.9±48	28.9	25.4	II *versus* III

BaP: benzo[a]pyrene; vit C: ascorbic acid; Se: selenium; GSH: glutathione; CP: carcinogenic potency; AP: anticarcinogenic potency

All reactive forms thus far characterized are electrophilic; this is probably the only connection among the structurally diverse chemical carcinogens. This theory was completed a few years later by Miller and Miller in the mid 1970s, who suggested that carcinogens or their metabolic products act as electrophiles initiating tumor formation through covalent interaction with nucleic acids or proteins of cells [17]. This hypothesis is now supported by recent investigations and by experimental findings where amines and other alkaline compounds can break the linkage of carcinogens to proteins, thus protecting the cells from carcinogenesis. A bulk of studies have been then reported on this topic.

The differences of susceptibility to carcinogenesis in many animals and humans may be explained, at least in part, by a biological inactivation mechanism (inhibiting the oncogenic action of carcinogenic agents) which exists in carcinogen-resistant animal species. This possibility is supported by the markedly reduced carcinogenic action of the strong carcinogenic agent BaP when it is injected in rabbits, pigs, or cows, in even higher concentrations. Similarly, certain physiologically occurring compounds, when administered to carcinogen-susceptible laboratory animals, effectively postpone or completely protect them from the oncogenic action of PAHs. These endogenous inhibitors of carcinogenesis can usually be classified into the following 3 groups: the first group includes intermediate products of the metabolic processes in human (animals) organisms; acids, amines, amino acids, thiols, vitamins, hormones, enzymes, porphyrins, chalones [4,18,19]. The second group includes foodstuff ingredients absorbed from the alimentary tract, e.g. compounds of the first group, as well as antioxidants, flavones, and metals [20]. And the third group includes metabolic products of microorganisms (bacteria and viruses) living permanently (mainly symbiotic in the intestine) or temporarily (pathogenic in tissues in humans, e.g. enzymes, antigens and cytostatic compounds) [21]. Besides the natural anticarcinogens, exogenous anticarcinogens also inhibit the PAHs-mediated carcinogenesis. Examples of synthetic anticarcinogens are quinones, peroxides and antioxidants [21].

Linus Pauling, Nobel Prize recipient, discussed the potential value of vit C for cancer prophylaxis, supportive therapy, and palliative treatment in advanced terminal cancer cases with a daily dose of 10 gr p.o. or intravenously; some beneficial responses of the regression type were registered [6]. Nonetheless, it is worth noticing that vit C has not received much attention in cancer research; relatively few articles were published on the effect of vit C in cancer prevention and treatment of neoplastic diseases. Fortunately, during recent years, a noticeable increase in interest has arisen for the use of vit C as a supplementary agent in cancer treatment.

Vit C restrains cell activities related to the enzyme hyalouronidase, which is released by cells and promotes their division, proliferation, and migration. Hyalouronidase liquefies and breaks down tissues. Proliferation continues as long as hyalouronidase is released and is stopped by physiological hyalouronidase inhibitors. There is evidence that a physiological hyalouronidase inhibitor is an oligoglycosaminoglycan that requires vit C for its synthesis. Thus, increased requirements for vit C that occur in many cell proliferation diseases including cancer may be explained. Therefore, vit C might be an effective drug for suppressing neoplastic cellular proliferation and invasiveness.

The early work on the use of vit C in cancer was emphasized since the middle of the 20th century. Nowadays, a definite connection between vit C and cancer both in favorable clinical data and in the detoxification of carcinogenic compounds has been shown in various cancer cases [22,23]. Likewise, its inhibitory effect on malignant tumors has been confirmed during the last two decades [24]. Furthermore, vit C prevents nitrosamine formation and inhibits tumor induction caused by carcinogens, such as aflatoxins or PAHs. There exists a paradox phenomenon that depends partially on the histological type of the tumor and was the reason for many controversial reports in scientific publications. Oral administration of high doses of vit C to tumor-bearing wistar rats is successful in decreasing the CP of BaP-induced fibrosarcomas and rhabdomyosarcomas, prolonging thus their survival.

This study showed the mean survival of G II to be significantly better when compared to G I (Table 1). Of note was the fact that one G II animal survived 820 days without any tumor development (a cyst was observed at the site of infection, instead of a tumor), reaching thus the normal lifespan. Tumor induction in G II was, therefore, 97.4%.

Several biologically active metals are inhibitors of PAHs carcinogenesis and are categorized as antitumor agents. The prophylactic as well as therapeutic effect of these metals depends on the amount present in the animal organism and the exogenic supply through food intake, drinks rich in appropriate minerals, and drug administration. The metals cobalt, zinc, vanadium, and selenium belong to the group of strong inhibitors of PAHs carcinogenesis [3,9-11,25]. A number of investigations demonstrating the prophylactic and inhibitory effect of Se on carcinogenesis have been reported [7,8,26], with low Se levels detected in patients

suffering from various types of cancer. Similarly, epidemiological data have shown that cancer mortality was significantly higher in geographic areas with low Se ground concentration compared with areas of high Se ground concentration; this is in connection with the Se concentration in the food and water.

Administration of 2 mg thiotic acid (lipoic acid) to EMAC cell-bearing animals led to a remarkable increase of the mitotic rate, in extreme cases up to 700%. We hypothesize that among the lipoic acid analogues, some inhibitors of tumor growth may be discovered. The fact that most agents known to initiate or retard cancer, react with sulphydryl groups, suggests an intimate relationship between cancer and –SH groups [13,27-29]. Theoretically, there are two possible ways to inhibit carcinogens with suitable synthetically prepared -SH derivatives reacting more selectively with the carcinogenic agents than the -SH groups of the cell constituents. This prevents the cell components from reacting with the carcinogens, and the carcinogens from possessing any carcinogenic potency. The second method is to block the -SH groups of vital cell constituents with compounds forming additional products with thiols. For example, unsaturated aliphatic acids, ketoaldehydes, etc. react with -SH groups of the cells, thus inhibiting their reaction with the carcinogens.

We have used GSH alone in current experiments in order to evaluate its inhibitory effect in tumor-bearing wistar rats. We have found that when GSH is administered alone it exhibits an AP of 10 units. The animals' mean survival was 225.6±30 days and the tumor incidence was 100%. For this reason we decided to use its combination with Se for more favorable results. We finally found that the combination Se+GSH exerts an AP of 25.4 units.

In conclusion, by this experimental work it was clearly shown that vit C is a good inhibitor of chemical carcinogenesis induced by BaP in tumor-bearing wistar rats. It was also shown that the combination of two anticarcinogens (Se+GSH) presents a significant antineoplastic effect, as was shown by the prolongation of survival in tumor-bearing wistar rats. Finally, administration of endogenous inhibitors like vit C, Se, and GSH, which were used in this study, can inhibit or modify the carcinogenic action of PAHs.

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