MICE LUNG ADENOMA BIOASSAY FOR STUDIES OF POSSIBLE MODIFYING AGENTS IN CARCINOGENESIS

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Abstract. Pulmonary tumours of mice are often been used as a tool in cancer research. The aim of this paper is to investigate and consider the utility of mouse pulmonary adenomas induced by urethane model in cancer chemoprevention studies.

Three sets of experiments have been conducted on inbred mice (both sexes), total – 372 animals. Potential anticarcinogenic compounds – vitamin E, novel organoselenium compounds – D-glucosamine hydroselenate and original anticarcinogenic mixture which consisted of these ingredients: retinol acetate, α-tocopherol, riboflavin, sodium selenite and glucuronic acid were given chronically per os, carcinogen – urethane was given by intraperitoneal injections. The experiments lasted for 4 months.

The results of our studies showed that all tested compounds exert inhibitory effect on lung carcinogenesis induced by urethane. Lung adenoma can serve as a model for analysis of complementary agents – cofactors whether intensifying or inhibiting pulmonary carcinogenesis.

Keywords: urethane, D-glucosamine hydroseleenate, vitamin E, original anticarcinogenic mixture, lung carcinogenesis, mice.

PELIŲ PLAUČIŲ ADENOMŲ MODELIS KANCEROGNEZĖS MODULIATORIŲ TYRIMAMS

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Trys eksperimentų serijos atliktos naudojant linijines peles (abiejų lyčių), iš viso 372 gyvūnai. Potencialūs antikancerogeniniai junginiai – vitaminas E, naujas organoselenio junginys D-gliukozamino hidroselenatas ir originalus mišinys, susidedantis iš retinolio acetato, α-tokoferolio, riboflavinio, natrio selenito ir gliukuronio rūgšties, buvo duodami pelėms per os, o kancerogenas uretanas švirkščiamas į pilvo ertmę. Bandymų trukmė – 4 mėnesiai.

Tyrimų rezultatai parodė, kad tirti junginiai slopino plaučių kancerogenezę, indukuotą uretana. Pelių plaučių adenomų modelis yra tinkamas tyrinėjant potencialius kancerogenės modifikuojančius (skatinančius ir slopinančius) veiksnius.

Raktažodžiai: uretanas, D-gliukozaminio hidroselenatas, vitaminas E, originalus antikancerogeninių veikimų pasižymėjusių medžiagų kompleksas, plaučių kancerogenezę, pelės.

Introduction. The new direction of cancer prevention – chemoprevention – is based on the knowledge of multistep pathway of carcinogenesis. This process may be interrupted by some chemical factors: vitamins, antioxidants etc. Cancer chemoprevention is an still experimental method, though it is gradually implemented in the clinical practice. Especially murine models are important in cancer research as they help to evaluate novel preventive and therapeutic strategies (Čespédes et al., 2006; Workman et al., 2010). Mouse models are useful of their relatively low maintenance cost, short gestation period, and ease of genetic manipulation and of the extensive information available on their genetic background (Nandan and Yang, 2007; De Jong and Maina, 2010).

Pulmonary tumours of mice have often been used as a tool in cancer research (Shimkin et al., 1966; Shimkin and Stoner, 1975; Roomi et al., 2009). L.L.Griciūtė was the
first who began these studies in Lithuania (Griciūtė, 1955).

It is known that the lungs of mice are especially responsive to urethane (ethyl carbamate). Pulmonary adenomas induced by this carcinogen often are multiple and their number depends on the dose of urethane used (Fig. 1, 2).

Fig. 1. Gross lung specimens of urethane-induced mice tumors

Adenomas may be found in either lung, in any lobe, are often situated just beneath the pleura and are recognized by their nodular, pearly, grey-white appearance contrasting with the more pink colour of the normal lung parenchyma.

Fig. 2. Urethane-induced mouse tumors, H&E

Adenomas are characterized by well-differentiated cuboidal cells with irregular round nuclei and clear to lightly eosinophilic cytoplasm forming glandular or papillary structures.

During the recent years, we carried out experiments using mice lung adenoma assay in studies of cancer modifying agents. The aim of this paper is to investigate and consider the utility of mouse pulmonary adenomas induced by urethane model in cancer chemoprevention studies.

Materials and methods. Animals: mice 8–10 weeks old, both sexes, and having a body weight of 20–24 g; mainly 20–30 (♂♀) animals in each group were used in the studies (total of 372 mice). The animals were obtained from the Animal Facility of State Research Institute Centre for Innovative Medicine (previous Institute of Immunology, Vilnius University). Experiment conditions were complying with Good laboratory practices and with the Law of the Republic of Lithuania on the Care, Keeping and Use of Animals as well as secondary legislation – Order of the State Food and Veterinary Service of the Republic of Lithuania “On Veterinary Regulations on Breeding, Handling and Transportation of Laboratory Animals” and “On the Use of Laboratory Animals in Scientific Experiments” (Law of the Care, Welfare and Use of Animals, 2002).

The animals were acclimatized for one week before the study; they were housed under constant conditions of temperature, humidity and light/dark cycle (12 h/12 h).

Exp. 1. Investigation of chemopreventive properties of vitamin E. The experiments have been carried out on BALB/C mice (142). The mice of this strain are of intermediate susceptibility for induction of lung tumours (Shimkin and Stoner, 1975). They were given urethane (commercial urethane, purum, Fluka, Buchs, Schweiz) in saline (100 µl) by intraperitoneal injections twice a week, single dose – 10 mg/mouse, total – 100 mg/mouse. Vitamin E (DL-α-Tocopherol, Merck, NJ, USA) 50 or 250 mg/kg b.w. was given by gavage (starting 1 week period before carcinogen injection) 3 times per week and thereafter during all the experiment (4 months).

Exp. 2. Investigation of chemopreventive properties of original novel selenium compound. D-glucosamine hydroselelante was synthesized in Institute of Oncology, Vilnius University. The experiments have been carried out on mice CBAxC57Bl (110). The mice of this strain are relatively resistant to induction of lung tumours (Shimkin and Stoner, 1975). They were given urethane by intraperitoneal injections twice a week, single dose – 10 mg/mouse, total – 50 mg/mouse. Selenium compound (1 or 10 mg/kg b.w.) was given per os chronically (dissolved in water, in aluminium foil-wrapped bottles to avoid light decomposition) ad libitum for 4 months.

Exp. 3. Investigation of chemopreventive properties of original anticarcinogenic mixture. The mixture was prepared in Department of Drug Technology of previous Kaunas Medical Institute (now – Lithuanian University of Health Science). The mixture consisted of the following ingredients in the relative amounts indicated: retinol acetate, 24 µg; tocopherol, 3.2 mg; riboflavin, 8.1 mg; sodium selenite, 3 µg and glucuronic acid, 9.8 mg.

The experiments have been carried out on BALB/c mice (120). These animals were purchased from Rapolov Animal Facility (St. Petersburg, Russia). Mice were given urethane as described in exp. 2. The duration of experiments was 4 months.
In all experiments body weights, food and fluid consumption and general health status were monitored weekly. After 4 months all surviving mice were killed by cervical dislocation. Mice were autopsied and examined. Excised lungs were fixed in neutral buffered 10% formalin. After fixation, all only macroscopically noted lung tumours were counted for histological verification of their adenomatous nature. Lungs were dehydrated in alcohols, embedded in paraffin, and 5-μ sections were stained with hematoxylin and eosin (H&E).

The results were statistically processed using Student’s *t*-test. Mean number of tumours per tumour-bearing mouse, percentage of animals with tumours, time to develop tumours, and their histological type were considered.

Results and discussion. As the aim of our studies was to adopt pulmonary adenomas bioassay for cancer chemoprevention studies we examined several potential chemical compounds.

Exp. 1. In the first set of experiments urethane induced lung adenomas in all mice, therefore we didn’t get the effect reflecting the influence of vitamin E on the incidence of adenomas (Table 1). However, it was found that vitamin E inhibited lung carcinogenesis induced by urethane: the average number of tumours per mouse was lower in comparison with a group given only carcinogen. We observed also dose-dependent effect: higher dose of vitamin E was more effective in comparison with lower dose (P ≤ 0.05).

Table. 1. Incidence and multiplicity of lung tumours in mice treated with urethane and vitamin E

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of animals</th>
<th>Number of animals with tumours (%)</th>
<th>Lung tumours/ mouse M±SD</th>
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<tbody>
<tr>
<td>Urethane</td>
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<td>♀</td>
<td>15</td>
<td>15 (100)</td>
<td>8.3±2.0</td>
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<tr>
<td>♂</td>
<td>14</td>
<td>14 (100)</td>
<td>11.7±3.9</td>
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<tr>
<td>Total</td>
<td>29</td>
<td>29 (100)</td>
<td>10.0±3.0</td>
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<td>Vitamin E + Urethane</td>
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<tr>
<td>♀</td>
<td>16</td>
<td>16 (100)</td>
<td>7.6±3.1</td>
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<td>♂</td>
<td>14</td>
<td>14 (100)</td>
<td>10.1±2.8</td>
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<tr>
<td>Total</td>
<td>30</td>
<td>30 (100)</td>
<td>8.8±2.9</td>
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<td>Vitamin E2 + Urethane</td>
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<td>15 (100)</td>
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<td>13</td>
<td>13 (100)</td>
<td>7.2±2.5</td>
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<td>Total</td>
<td>28</td>
<td>28 (100)</td>
<td>7.0±2.7 *</td>
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<td>Vitamin E1</td>
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<td>Total</td>
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Vit. E1 – 50 mg/kg b.w.; Vit. E2 – 250 mg/kg b.w.; * P ≤ 0.05

During the last decades, scientists have been focused in the role of vitamin E in cancer (reviewed in Mamede et al., 2012). Though many of these studies are contradictory, several studies show that vitamin E could be an important agent against lung cancer (Quin et al., 2005; Yang et al., 2010; Li et al., 2011). The anticarcinogenic activity of vitamin E may be exerted by several mechanisms. It is known that vitamin E has an antioxidant effect, is involved in the biosynthesis of heme and proteins, cell proliferation, tissue respiration, and other critical processes of tissue metabolism; vitamin E inhibits free radical reactions, prevents the formation of peroxides that damage cellular and subcellular membranes (Shibata et al., 2008; Baumeister et al., 2009; Comitato et al., 2009). Our previous studies showed the protective effect of vitamin E on benzo(a)pyrene induced chromosome damage in mice bone marrow cells (Slapsyte et al., 2011).

Exp. 2. In the second set of experiments we used lower dose of carcinogen (in comparison with exp. 1) and strain of mice which is less responsible to induction of lung tumours. Chronic oral consumption of novel organoselenium compound was effective against urethane induced lung carcinogenesis: D-glucosamine hydroselenate reduced multiplicity of tumours per mouse by 44–50% (p < 0.05), as well as inhibited incidence of tumours as shown in Fig. 3.

![Fig. 3. Incidence of lung adenomas in mice given urethane (U) and D-glucosamine hydroselenate (D)](image-url)

D*—1 mg/kg b.w., D**—10 mg/kg b.w. (per os)

The majority of animal and clinical studies as well as our studies show selenium compounds (both organic or inorganic) as cancer chemoprevention agents, the efficacy of which depends on the stage of carcinogenesis, chemical form of selenium, formulation and dosages (Schrauer, 2000; Uleckienė et al., 2005; Jackson and Combs, 2008; Yan and DeMars, 2011). Selenium exerts its anticarcinogenic effect by multiple mechanisms. It is known that some of the glutathione peroxidases, which are antioxidant enzymes, require the presence of selenium as an essential cofactor, thus suggesting that low selenium intake impairs antioxidant defences. Other mechanisms for selenium and its compounds anticarcinogenicity...
include: alteration of carcinogen metabolism, cell cycle regulation, immune surveillance, cancer cell migration and angiogenesis, cell death programming (Jackson and Combs, 2008).

Exp. 3. The results of studies showed that original mixture (retinol acetate, 24 µg; α-tocopherol, 3.2 mg; riboflavin, 8.1 mg; sodium selenite, 3 µg and glucuronic acid, 9.8 mg) statistically significant reduced multiplicity of lung adenomas induced by urethane. The inhibitory effect was 20 % (p<0.05). Our previous studies have demonstrated that this mixture inhibits renal tumours induced by N-nitrosodimethylamine in rats. The similar anticarcinogenic effect was observed in animal models using other carcinogens (N-nitrososarcosine, N-metil-N-benznilnitrosamine, 7,12-dimethylbenzantracene) (personal communication).

The effect of dietary intake of various mixtures of nutrients is widely studied in clinical trials as well as in experiments in vivo (Zhang et al., 2008; Olsen et al., 2009; Park et al., 2010; Black, 2010; Molnar et al., 2012). Recently new data on the effect of nutrient mixtures on lung tumorigenesis have been published. For instance, it was shown that nutrient mixture consisted of several ingredients – vitamin C (as ascorbic acid and as Mg, Ca, and palmitate ascorbate), L-lysine, L-proline, L-arginine, N-acetylcysteine, standardized green tea extract, selenium, cooper, manganese has inhibitory potential on the development of mouse lung tumours induced by urethane (Roomi et al., 2009). Inhibition of lung cancer growth was observed in mice given dietary tocopherols (Lambert et al., 2009). In the other studies using various models the inhibitory effect of γ-tocopherol-rich mixture of tocopherols against lung tumorigenesis and the growth of xenograft tumours of human lung cancer cells were demonstrated (Lu et al., 2010). On the other hand, combined treatment with quercetin and vitamin E did not demonstrate any effect greater than that due to vitamin alone in smoking-induced lung tumour in mice (Yang et al., 2008). Further studies are necessary to determine the underlying mechanisms for the anticancer activity if such activity occurs in other models of cancer.

In general, in three sets of our experiments urethane-challenged mice developed only benign lung tumours. Histological examination revealed adenomas which were characterized by well-differentiated cuboidal cells with irregular round nuclei and clear to slightly eosinophilic cytoplasm, forming glandular or papillary structures. No tumours were evident in the livers, kidney, and spleen in any of the mice.

Thus, what could be learned from these experiments? Firstly, we can mention that the induction of primary pulmonary tumours is relatively rapid; with highly active carcinogen, such as urethane, grossly visible nodules can be identified on the surface of the lungs within 3 months, although longer periods are preferred in order that the tumours may attain larger size. The tumours are easily recognized and have a typical appearance grossly and under the microscope. The multiplicity of tumours, directly related to the dose of the carcinogen, is an important quantitative dividend.

There are, of course, certain real or assumed drawbacks to the pulmonary tumour bioassay technique. Perhaps the most important ones are the belief that these are not truly malignant tumours and those they have no close counterpart in human pathology. Since with the more susceptible strains, all mice eventually develop such tumours (Shimkin et al., 1966).

According to the literary data, in recent years the mouse lung adenomas model has been mainly used for investigation of cancer chemopreventive properties of various agents, for instance: N-acetylcysteine, 2-amino-2-thiazololzine salt (Simkeviciene et al., 2002), myelopropide-2 (Mikhailova et al., 2002), β-carroene (Uleckiene and Domkiene, 2003), selenium compounds (Uleckiene et al., 2005), melatonin (Vensushkin et al., 2006), original nutrients mixtures (Roomi et al., 2009), β-glucan (Zabulyte et al., 2012).

All studies have been conducted on mice, and although the animal lung cancer mirrors human lung cancer, there is usual limitation regarding translation of animal model to practical use in humans. During last decade, a rapid progress in developing new animal models for lung cancer chemoprevention studies was observed (Zheng and Takano, 2011). However, lung adenoma assay is a classical example and could depend on selection of the question we are planning to answer. Due to evaluation of our previous investigations, we are continuing our studies using urethane-induced lung tumour model for assessment of possible cancer modifying agent such as sodium valproate.

Conclusions.
1. All tested compounds showed cancer chemopreventive activity: vitamin E, novel organoselenium compound-D-glucosamine hydroselenate and original anticarcinogenic mixture (retinol acetate, α-tocopherol, riboflavin, sodium selenite, and glucuronic acid) were found to inhibit lung adenomogenesis in mice.
2. Mice lung adenoma model is useful to assess the efficacy of potential chemopreventive agents.

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Literature.


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