Joint Effects of Cigarette Smoking and Irradiation
in Pregnant Mice and their Offspring

SÁRA ANTAL1, BÉLA SZENDE2, JÓZSEF LENGYEL3 and EGON J. HIDVÉGI1,4

1Radiobiological Foundation, Budapest, 1221 Ringló str.89;
2First Department of Pathology and Experimental Cancer Research and
3Central Isotope Laboratory, Semmelweis University, Budapest,1085 Üllői str.26;
4National Research Institute for Radiobiology and Radiohygiene, Budapest,1221 Anna str.5, Hungary

Abstract. Background: We have previously reported that irradiation of mice in utero significantly increased the tumor incidence in the offspring of irradiated mothers. The joint effects of irradiation and cigarette smoking (CS) on tumor incidence and on the process of carcinogenesis were investigated. Materials and Methods: Pregnant C57Bl/6J female mice were irradiated with a single dose of γ-ray (1Gy or 3Gy) and/or exposed to CS of IR3 non-filtered cigarettes before or during pregnancy. Tumors were investigated both with histological and immunohistochemical methods. Results: Longer exposure (60 days) of the mice to CS before pregnancy and irradiation during pregnancy significantly increased the tumor incidence in the mothers and their offspring. Parallel activation of Caspase-8 and inactivation of Caspase-9 was found. Conclusion: Joint exposure of mice to prolonged CS before pregnancy and irradiation during pregnancy significantly increased the tumor incidence both in the mothers and their offspring.

In the last decades lung cancer has become a leading type of cancer both among men and women as a consequence of the elevated risk in the growing number from people with a smoking habit. In 2002 the mortality rate of lung cancer among men in Central Europe was highest in Hungary (103 age-standardized rate per 100,000 capita). The lung cancer mortality among women was 29 age-standardized rate per 100,000 capita vs. 27.7 for breast cancer (1). One of the first examinations of tobacco smoking and another chemical exposure (i.e. asbestos) was published by Saracci (2, 3), who observed that asbestos and smoking independently produced lung cancer in humans and that they acted synergistically when exposure to both occurred. We have already reported that after irradiation of mice embryos, in utero, a significant increase of tumor incidence was observed during the adult life of the offspring as an effect both of γ-ray (20-25%) and fission neutrons (40-60%), as compared to the low incidence in unirradiated-control mice (18%) (4). In this study the tumor incidence in pregnant female mice and their offspring after exposure to γ-irradiation alone, cigarette smoking (CS) alone or a combination of the two was investigated. Detailed morphological and immunohistochemical descriptions of the resulting tumors are presented.

Materials and Methods

Animals. Two to three month old, 22-24g weight, C57Bl/6J female and DBA2 male mice were used (Charles River Ltd., Budapest, Hungary). All the animal studies were approved by the local Ethics Committee (Semmelweis University). The female C57Bl/6J mice were mated with the DBA2 male mice for five days. The average gestation time was 19 days and the average litter size was 7. Since the number of offspring was over 100/group with slight variations the animals were randomized and the tumor incidence was expressed as percent.

Experimental groups. The following treatments were applied, to the female mice (groups I-II) and their offspring were recorded as groups I. a to II. a: untreated control (group 1); females exposed to smoke for ten days before pregnancy (group 2) females exposed to smoke for ten days during the first part of pregnancy (group 3); females exposed to smoke for ten days during the second part of pregnancy (group 4); females exposed to 1Gy irradiation during the second part of pregnancy (group 5); females exposed to smoke for 60 days before pregnancy (group 6); females exposed to 3Gy (group 7); females exposed to smoke for 10 days during the first part of pregnancy and 1Gy irradiation during the second part of pregnancy (group 8); females exposed to smoke for 10 days and irradiated with 1Gy during the second part of pregnancy (group 9); females exposed to smoke for 10 days and irradiated with 3Gy during the second part of pregnancy (group 10); and females exposed to smoke for 60 days before pregnancy and irradiated with 1Gy during the second part of pregnancy (group 11).
Animal care. The animals were placed in double-sided, ventilated racks (IFFA CREDO, Paris, France). Rodent chow (Charles River Ltd.) and water were given ad libitum and the animals were observed twice daily.

Cigarette smoking (CS). IR3 (code name) non-filtered cigarettes were purchased from the Tobacco and Health Research Institute (Lexington, Kentucky, USA). The experimental smoke exposure machine (ESEM) for whole body exposure of the mice was designed for this program by J. Naményi, Ph.D. (Medi-Care-Sys Ltd. Budapest, Hungary). The program of ESEM provided CS under defined conditions. The computer program of the exposure to CS simulated the conditions of human CS. The mice inside the smoking chamber were placed in a specially prepared cage. The smoke from 15 cigarettes was mixed 1:10 in air. In a typical one minute cycle a puff of two seconds duration was followed by a 30 seconds smoke hold period for a total exposure and a 30 second purge cycle was used to clear out of smoke. The cycle was then repeated. The mice were exposed to CS twice daily, for five days per week. The control animals were sham exposed without cigarette smoke. The efficiency of CS was monitored by assaying cotinine, one of the main metabolites of nicotine. Analysis was performed 2-16 times from urine samples during the smoking period by the method of Oddoze et al. (5). The cotinine level in the CS exposed animals was between 608-1048 μg/ml.

Irradiation. In the second part of gestation, the mice were irradiated with a single dose γ-ray (1Gy, 3Gy) by Gammatron-3 (Siemens, Erlangen, Germany), dose rate: 0.1789 Gy/min.

Tumor incidence and histological examinations. The mice were sacrificed when they were looking visibly ill or at the end of the second year of age. The organs were removed, fixed in 8% formalin, embedded into paraffin and 8μ thin sections were cut. The sections were stained with hematoxylin and eosin (HE). In selected cases immunoperoxidase reactions were performed. An Apop-Tag assay- TUNEL reaction (Q Biogene obtained from Biomarker, Gödöllő, Hungary) was used for the detection of apoptotic cells in the tumors. Antibodies to P53, Bcl2, Bax (Dako, Glostrup, Denmark), Caspase 8, Caspase 9, CD3, CD10 and c-myc (Novocastra, Newcastle, UK) were used for immunohistochemical investigations. Because of the use of mouse monoclonal antibodies a „mouse on mouse” (mom) kit was applied to avoid non-specific reactions (Vector Laboratories Burlingame, CA, USA). Diaminobenzidine served as the chromogen (Vector Laboratories Burlingame, CA, USA). The SPSS 11.5 statistical program (SPSS Inc. Chicago, USA) was used.

Results

Tumor incidence. Table I shows the tumor incidence in the female mice after exposure to CS only or CS and/or irradiation during pregnancy. If the animals were exposed to CS only for 10 days, (groups 2, 3 and 4) the tumor incidences were about the same as in the unexposed controls (group 1). However, if the animals were exposed to CS for 60 days before pregnancy (group 5) the tumor incidence more than doubled (40%) compared to the unexposed control level (18%).

If the animals were irradiated with 1Gy only, during the second part of pregnancy and exposed to CS either in the first part of pregnancy (group 8) or in the second part (group 9) the tumor incidence was about the same as in the only irradiated group (group 6). Significantly higher tumor incidence (42%) was found when the CS exposure was in the second part of pregnancy, but the radiation dose was increased to 3Gy (group 10). Interestingly, if the mice were exposed to CS for 60 days before pregnancy and exposed to 1Gy γ-irradiation during the second part of pregnancy (Group 11) a higher tumor incidence (47%) was found compared to the other combinations (group 8, 29%, group 9, 24%). Table I shows that 28% tumor incidence was found after 1Gy (group 6) and 32% after 3Gy γ-irradiation (group 7) of the mice during the second part of pregnancy. Table II shows the tumor incidence of the offspring. The incidence of tumors was similar to that in their mothers. Longer CS exposure of the mice before pregnancy either alone or jointly with irradiation significantly increased the tumor incidence (groups 5: a and 11a).

Histological examinations Lymphomas: According to the cell surface markers the majority of lymphomas (23) were of B cell origin (groups 1-11, Figure 1). Only three T cell lymphomas were found (groups 5, 7, 9). Two lymphomas showed neither CD10, nor CD3 positivity. The cell morphology and tissue pattern pointed to lymphocytic lymphoma or follicular centre cell lymphoma.

Chronic myeloid leukemia (CML): At autopsy, marked enlargement of the spleen and liver was observed. These organs were diffusely infiltrated by granulocytic cells of intermediate maturity and also by mature granulocytes.

Lung adenoma and carcinoma: The benign tumors were sharply circumscribed nodules composed of fairly uniform, closely packed columns of cuboidal or columnar cells arranged in tubular of papillary structures with scanty fibrovascular stroma. The pulmonary carcinomas showed dedifferentiation and cellular pleomorphism with invasion of lung parenchyma and occasionally intrabronchial growth (Figure 2).

Ependymoma: The two ependymomas (groups 9, 11) were malignant growths protruding into the ventricles of the brain, composed of differentiated ependymal cells. Occasionally pseudo rosette formation was observed (Figure 3).

Glioma: The glioma (group 3) showed histologically the characteristics of astrocytoma.

Angiomaticus lesions of the liver: These lesions presented true cavernous hemangiomas.

Leiomyoma and leiomyosarcoma of the uterus: These tumors were composed of spindle cells showing smooth muscle differentiation. The majority of the uterine tumors fulfilled the criteria of leiomyosarcoma.
The following sporadic tumors showed the characteristics known from textbooks: granulosa and thecal cell ovarian tumor, islet cell adenoma of the pancreas, papilloma of renal pelvis, duodenal adenoma, adenocarcinoma of the rectum, retroperitoneal sarcoma, papilloma and squamous cell carcinoma of the skin, hemangioma of the skin, fibrosarcoma of the skin, conjunctival papilloma, papillary adenoma of the lacrimal gland and neuroma.

Table I. Tumor incidence in female mice after joint exposure to cigarette smoking and γ-irradiation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Exposures</th>
<th>γ-ray, dose (2)</th>
<th>Number of animals</th>
<th>%</th>
<th>Frequent tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Unexposed-control</td>
<td></td>
<td>4/27</td>
<td>18</td>
<td></td>
<td>Lymphoma</td>
</tr>
<tr>
<td>2 for 10 days before pregnancy</td>
<td></td>
<td>5/20 ns. vs. 1</td>
<td>25</td>
<td></td>
<td>Lymphoma</td>
</tr>
<tr>
<td>3 for 10 days in the first part of pregnancy</td>
<td></td>
<td>5/22 ns. vs. 1</td>
<td>23</td>
<td></td>
<td>Lymphoma, Lung Adenocarcinoma</td>
</tr>
<tr>
<td>4 for 10 days in the second part of pregnancy</td>
<td></td>
<td>4/20 ns. vs. 1</td>
<td>20</td>
<td></td>
<td>Lymphoma</td>
</tr>
<tr>
<td>5 for 60 days before pregnancy</td>
<td></td>
<td>8/20 s. vs. 1</td>
<td>40</td>
<td></td>
<td>Lymphoma, Lung Adenocarcinoma, Skin Tumor</td>
</tr>
<tr>
<td>6 -</td>
<td>1 Gy</td>
<td>7/25 s. vs. 1</td>
<td>28</td>
<td></td>
<td>Lymphoma, CML</td>
</tr>
<tr>
<td>7 -</td>
<td>3 Gy</td>
<td>8/25 s. vs. 1</td>
<td>32</td>
<td></td>
<td>Lymphoma, Lung Adenocarcinoma</td>
</tr>
<tr>
<td>8 for 10 days in the first part of pregnancy</td>
<td>1 Gy</td>
<td>6/21 ns. vs. 6 s. vs. 1</td>
<td>29</td>
<td>Lymphoma, Lung Adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>9 for 10 days in the second part of pregnancy</td>
<td>1 Gy</td>
<td>5/21 ns. vs. 6 s. vs. 1</td>
<td>24</td>
<td>Lymphoma, Lung Adenoma</td>
<td></td>
</tr>
<tr>
<td>10 for 10 days in the second part of pregnancy</td>
<td>3 Gy</td>
<td>10/24 s. vs. 6 s. vs. 4</td>
<td>42</td>
<td>Lymphoma, Lung Adenocarcinoma, Leiomyosarcoma</td>
<td></td>
</tr>
<tr>
<td>11 for 60 days before pregnancy</td>
<td>1 Gy</td>
<td>9/19 s. vs. 6 s. vs. 7 s. vs. 1</td>
<td>47</td>
<td>Lymphoma, Lung Adenocarcinoma</td>
<td></td>
</tr>
</tbody>
</table>

(1) CS: Cigarette smoking, (2) irradiation in the second part of pregnancy, ns.: non-significant, s.: significant, \( p<0.05 \), CML: chronic myeloid leukemia.

Table II. Tumor incidence in mice after joint exposure to cigarette smoking and γ-irradiation, in utero.

<table>
<thead>
<tr>
<th>Group</th>
<th>Exposures</th>
<th>γ-ray, dose (2)</th>
<th>Number of animals</th>
<th>%</th>
<th>Frequent tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.a Unexposed control</td>
<td></td>
<td>16/100</td>
<td>16</td>
<td></td>
<td>Lymphoma</td>
</tr>
<tr>
<td>2.a for 10 days before pregnancy</td>
<td></td>
<td>20/100 ns. vs. 1</td>
<td>20</td>
<td>Lymphoma</td>
<td></td>
</tr>
<tr>
<td>3.a for 10 days in the first part of pregnancy</td>
<td></td>
<td>18/100 ns. vs. 1</td>
<td>18</td>
<td>Lymphoma, Lung Adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>4.a for 10 days in the second part of pregnancy</td>
<td></td>
<td>21/100 ns. vs. 1</td>
<td>21</td>
<td>Lymphoma</td>
<td></td>
</tr>
<tr>
<td>5.a for 60 days before pregnancy</td>
<td></td>
<td>38/100 s. vs. 1</td>
<td>38</td>
<td>Lymphoma, Lung Adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>6.a -</td>
<td>1 Gy</td>
<td>26/100 s. vs. 1</td>
<td>26</td>
<td>Lymphoma, Lung Adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>7.a -</td>
<td>3 Gy</td>
<td>28/100 s. vs. 1</td>
<td>28</td>
<td>Lymphoma, Lung Adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>8.a for 10 days in the first part of pregnancy</td>
<td>1 Gy</td>
<td>30/100 ns. vs. 6, s. vs. 1</td>
<td>30</td>
<td>Lymphoma, Liver tumor</td>
<td></td>
</tr>
<tr>
<td>9.a for 10 days in the second part of pregnancy</td>
<td>1 Gy</td>
<td>27/100 ns. vs. 6 s. vs. 1</td>
<td>27</td>
<td>Lymphoma, CML</td>
<td></td>
</tr>
<tr>
<td>10.a for 10 days in the second part of pregnancy</td>
<td>3 Gy</td>
<td>40/100 s. vs. 6 s. vs. 4</td>
<td>40</td>
<td>Lymphoma, Lung Adenocarcinoma, Ovarium tumor</td>
<td></td>
</tr>
<tr>
<td>11.a for 60 days before pregnancy</td>
<td>1 Gy</td>
<td>45/100 s. vs. 6 s. vs. 7 s. vs. 5</td>
<td>45</td>
<td>Lymphoma, Lung Adenocarcinoma, Liver tumor CML</td>
<td></td>
</tr>
</tbody>
</table>

(1) CS: Cigarette smoking, (2) irradiation in the second part of pregnancy, ns.: non-significant, s.: significant, \( p<0.05 \), CML: chronic myeloid leukemia.
Immunohistochemical examination. TUNEL positivity varied between 1 and 4% in 50% of the investigated cases and was below 1% in the other half. Three of the leiomyosarcomas and the ependymoma showed nuclear positivity of P53 to an evaluable degree. Other tumors were P53 negative. The lymphomas were Bax negative. The majority of lymphomas showed low (5-10%) cytoplasmic Bcl2 positivity.

Caspase 8 and 9 were investigated in the lymphomas and cytoplasmic caspase 8 positivity was found in all of them to various degrees (5-60%). All the lymphomas were Caspase 9 negative. The ependymomas and the glioma showed more than 80% nuclear c-myc positivity. The leiomyosarcomas revealed focal positivity in several groups of tumor cells (Figure 4). The lung adenocarcinomas, lung adenomas and skin carcinomas were negative for c-myc.

Discussion

The authors have previously reported that the tumor incidence significantly increased (23-35%) in adult mice after a single 0.5-2.0 Gy dose of γ-irradiation \textit{in utero} (6). An increased tumor incidence was also observed after 0.5 Gy fission neutron irradiation as compared with the low tumor incidence of unirradiated control mice (40-60% vs. 18%) (4). In the present study the joint exposure to CS and γ-irradiation produced significantly elevated tumor incidence not only in the female mice but also in the offspring (Tables I and II, groups 10,10a and 11,11a). After a long period of CS exposure (60 days) before pregnancy and 1 Gy γ-radiation during the second part of pregnancy the tumor incidence more than doubled (group 11, 11a) compared with untreated
control animals (group 1, 1a). The histological results demonstrated the appearance of some rare tumors in the CS exposed groups including two ependymomas and one glioma. We have never previously observed spontaneous brain tumors in unexposed mice (total 1009 examined) (6-8).

A-bomb survivors have proved that ionizing radiation significantly increases the incidence of malignant diseases. By the end of 2000, 47,529 out of 86, 611 cohort members had died and 10,085 of these deaths were caused by solid cancer. Nineteen percent of the solid cancer deaths occurred in the last 7 years (9, 10). Increased cancer mortality was also observed in prenatally exposed survivors (13, 14). In another study (15) estimated that a cumulative exposure of 100mSv would lead to 10% increased mortality from all malignancies. However it was noted that less than 5% of workers received this dose during their lifetime. The study attempted to take into consideration, not only the dose of radiation, but also the smoking habit, although unfortunately, the particulars of smoking habit were uncertain (15). As a consequence of the Chernobyl accident more than two-million women of reproductive age are living in the slightly radioactive polluted area around Chernobyl. Approximately a half of them have smoked even they were pregnant (16). Petridou et al. (17) reported that infants exposed to ionizing radiation in utero from fallout in Greece experienced a 2.6 times higher incidence of leukemia compared to unexposed children.

Epigenetic studies have indicated that different pathways are involved in the carcinogenic process in smokers and the non-smoker population (18-20). It is relevant to mention an earlier study from this laboratory which showed that in γ-ray induced mouse adenocarcinomas probably different pathways were activated then in spontaneous ones (4, 6). Therefore if might be supposed that in the course of joint exposure of an organism to γ-ray and CS at least three different pathways are involved in lung carcinogenesis. Although c-myc expression has been found in lung tumors of mice irradiated in utero (6) in the present study the lung tumors of animals exposed to CS failed to express this oncogene. However, c-myc expression was detected in the brain tumors and leiomyosarcomas in the uterus.

In the present study apoptotic activity was relatively low in all the investigated tumors. In all the tumors Bax was found to be negative and most were moderately Bcl2 positive. The negative results obtained after P53 immunostaining suggested that the apoptotic pathway in the observed tumors was P53 independent. All the investigated tumors were Caspase 9 negative and most of them were Caspase 8 positive. These observations suggested that in the final stage of tumorigenesis the plasma membrane associated death receptor pathway became activated and not the mitochondrial route.

Kuo and his coworkers (21) investigated the role of CS on the very early stage of lung adenocarcinogenesis and found that the mitochondrial pathway of apoptosis was activated. This, however, does not contradict the present results since this investigation of apoptosis was in already developed lung tumors where other pathways of apoptosis might be activated than during the course of carcinogenesis.

Acknowledgements

We express our gratitude to the Radiobiological Foundation (Budapest, Hungary) for managing the project and supporting the construction of the smoking machine. We thank Prof. Kálmán Magyar, MD, Ph.D., DSc. (Semmelweis University, Budapest) for his suggestions. We thank Ms. Éva Tölcsér and Ms. Éva Fejes for their valuable assistance in the smoking and radiation exposure. We thank Ms. Katalin Paczolay, Ms. Erzsébet Kiss and Ms. Mária Cserneké for their excellent histological assistance. Research described in this article was supported in part by Philip Morris USA, Inc. and by Philip Morris International.

References


Received August 8, 2008
Revised December 29, 2008
Accepted January 27, 2009