UNIVERSITY OF TARTU

ADRENALS AROUND CARCINOGEN CONTAINING IMPLANTS SUPPRESS TUMORIGENESIS

MASTER THESIS

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LIST OF ORIGINAL PUBLICATIONS

I. Mesila I. Biological material around DMBA implants reduces tumorigenesis. Papers on Anthropology 2001; X: 178-190.

II. Mesila I. Mitotic activity in intact organs of rats affected by DMBA implants in the abdominal cavity. Papers on Anthropology 1999; VIII: 127-145.

III. Mesila I. Reactive histological changes in DMBA-containing implants grafted into enucleated adrenals of rats. A. Rauberi mälestuskonverents, Tartu Ülikool, 1991: 37-48.

ABBREVIATIONS

DMBA	7, 12 - dimethylbenz(a)anthracene
DAB	3, 3´ - diaminobenzidine
MI	mitotic index
fs	fibrosarcoma
rms	rhabdomyosarcoma
adc	adenocarcinoma
pleos	pleomorphic cell sarcoma
sc	scirrhus
lipos	liposarcoma
n	number
Fig	figure

1. INTRODUCTION

Experimental carcinogenesis by using 7,12-dimethylbenz(a)anthracene (DMBA) is well known. The DMBA-induced rat tumour model has found a broad application as a tool in the preclinical evaluation of drugs (Steele et al., 1994). Small doses of cancerogenic substances given during a long term can cause cancer much more probably than a single large dose. Therefore various substances must be added to carcinogenic stuff to slow down the release of carcinogenic material and cause more tumours during the experiment. A few authors have transplanted DMBA also in biological tissue material (Shiba et al., 1982; Nishida et al., 1998). Arousal of different types of tumours due to DMBA has been described but we could not find any literature, comparing results of tumour genesis and reactive changes with and without biological material.

DMBA has an effect on cell proliferation and mitotic activity is studied in connection with tumour genesis. Mitotic index (MI) is a good indirect measure indicator of cell proliferation. Cell proliferation itself has been demonstrated to be a strong predictor of outcome for tumour (Romansik et al., 2007). MI rises inside the tumour but there is no data about mitotic activity in intact organs by presence of tumour background in organism.

Lymphocytes are involved in antitumour immunity. Inflammatory changes have been described in DMBA tumours and found an inverse relationship has been found between the number of lymphocytes and tumour formation.

The main goal of the present thesis was to study the effects of biological tissue on the development of tumours and variation of reactive changes in DMBA-containing implants and surrounding tissue, accompanying changes in mitotic activity in intact organs.

2. REVIEW OF THE LITERATURE

2.1. DMBA models

DMBA is a synthetic, polycyclic aromatic hydrocarbon, which is used as a prototype chemical carcinogen due to its several carcinogenic effects. Experimental DMBA induction of carcinogenesis is thoroughly investigated with the use of special experimental models. DMBA can be administered intragastrically through a probe (Albright et al., 1982; Ether et al., 1982; Frankov, 1980; Hultborn et al., 1993; Mc Gaughey et al., 1982; Mc Gaughey et al., 1983; Schweicer et al., 1987; Vengadesan et al., 1998; Yoshida, 1983), by local coetaneous and mucosal application (Mobil et al., 2006; Girard et al., 2001; Kessler et al., 1983; Türctenberger et al., 1983) and pouch mucosal painting (Chen et al., 2003), by bronchoscope submucosal treatment (Lavi et al.,1982), intravenously (Yoshiaki et al.,1982; Sylvesrter et al., 1982), orally with food (Blanco-Aparicio et al., 2007; Mathivadhani et al., 2007; Uppala et al., 2005; Chen et al., 2005; Manjanatha et al., 2005; Brandes et al., 1992; Klamer et al., 1983; Loscher et al., 1997), by subcutaneous injections (Fujiwara et al., 1994), by intraperitoneal injections (Ozturk et al., 2002), by local intraductal mammary injections (Tekmal et al., 1997; Tereda et al., 1994), by intramuscular injections into the neck region (Whitmire et al., 1978), by injection into the salivary gland (Wang et al., 1998). Different methods for implanting carcinogenic foreign substances (Brayan, 1969) and transplants in subcutaneous tissue (Reiners et al., 1997; Shiba et al., 1982), in the spleen (Nishida et al., 1998), in sublingual submucosa (Fathy, 1993), in the bladder (Ball et al., 1964), in the pylorus (Soloven et al., 1963), and in trachea (Kendrick et al., 1974) have been described in experimental research.

2.2. Reducing the speed of carcinogen release

Carcinogen pellets have been made using gelatine (Kendrick et al., 1974) or wax (Ball et al., 1964; Griesmer et al., 1975). Some authors have found that small doses of carcinogenic substances applied during a long term can cause cancer much more efficiently than a single large dose given on one occasion (Goodall et al., 1970; Taylor et al., 1975). Therefore various soluble and insoluble materials including lipid matrices, particularly stearyl alcohol, cholesterol (Rubin et al., 1977; Shiba et al., 1982; Veal et al., 1976) and carbon particles (Brayan 1969; Shiba et al., 1982; Rubin et al., 1977) have been added to carcinogenic substrates to reduce the speed of polycyclic hydrocarbons release into the tissue.

A few authors have transplanted DMBA with biological material such as the trachea (into subcutaneous pocket) (Shiba et al., 1982) or ovarian tissue (into spleen) (Nishida et al., 1998). *Shiba et al.* demonstrated that a higher incidence of dysplastic and neoplastic lesions was induced in the tracheal mucosa when the rate of release of DMBA had been lowered (Shiba et al., 1982). To produce malignant granulosa cell tumours, *Nishida et al.* autografted ovarian tissue, containing DMBA, into the spleen (Nishida et al. 1998).

2.3. DMBA induced tumours

Tumours which arise after DMBA application originate from epithelium – carcinomas (Shiba et al., 1982; Soloven et al., 1963) and also from mesenchyma – sarcomas (Binz et al., 1983; Fathy, 1993; Galton et al., 1982; Hažialov et al., 1986; Nishida et al., 1998; Vasilyev et al., 1988;). DMBA can induce tumours in different organs and tissues. Mammary tumours are most frequent and known by gavages orally administrated or injected DMBA and skin or mucosal tumours arise by local applications and paintings. Different tumours are found in liver, lung (McDermott et al., 2007; Chen, et al., 2005), stomach, ovary (Blanco-Aparicio et al., 2007), salivary gland (Wang et al., 1998) and sublingual gland (Takeuchi et al.,

1975) either both myeloid and lymphoid lineages lymphomas (Wang et al., 1998) and leukaemia (Huggins et al., 1966).

2.4. Reactive changes

Usually lymphocytes are involved in antitumour immunity (Ruggiero et al., 1989) and the intensity of immunologic reactions can affect the destiny of cell transformation (Umanski, 1975). Apart from the well-known tumourigenic effect and cell proliferation stimulating effect, DMBA also causes inflammation (Brandes et al., 1991). Inflammatory changes have been described in DMBA models before the development of tumours and an inverse relationship between the number of lymphocytes and tumour formation has been found (Heppner et al., 1988). On the other hand, in the process of foreign body tumourigenesis the inflammatory reaction is caused by the presence of a foreign body and it usually inhibits tumour formation (Brand et al., 1975).

2.5. Mitotic activity

Mitotic activity is one of the well-investigated indicators of cell proliferation. Cell proliferation and mitotic activity have been studied in the relationship with chronobiology (Dobrohotov et al., 1962; Krasilnikova, 1962; Brayan, 1969; Orlova, 1962; Romanov et al., 1994; Romanov et al., 1970), ageing (Romanov et al., 1969), antibacterial treatment (Romanov et al., 1994), interleukin treatment (Caporale et al., 2007), infection (Romanov et al., 1994), feeding effect (Claus et al., 2006), influence by haemopoietic growth factors (Earle et al., 2007), as observed in small intestine (Claus et al., 2006), oesophagus and intestine, in hyperinsulinemic liver (Dombrowski et al., 1995), in tumour by stress (Steplewski et al., 1985), in liver by x-ray (Kropachova et al., 1981), in spleen (Caporale et al., 2007), by tumour formation (Vemireddi et al., 2007). Many authors have investigated manifestations of carcinogenic and co-carcinogenic activity in the origin and growth of tumours. Local cell proliferation increases and so does mitotic activity, which is measured and

expressed by a rise in the proliferation index or the mitotic index (Bednarek et al., 1997; Begwe et al., 1994; Brandes et al., 1991; Ramchandani et al., 1998; Schultz et al., 1989; Tsambaos et al., 1989). Some scientists have reported on treating tumours with inhibitory chemicals having antiproliferation and antimitotic effect. The effect results in decrease and inhibition of cell proliferation and mitotic activity as well as regression of the tumour. The slowing of the tumour growth is largely due to the reduced mitotic activity (Claus et al., 2006; Blanco-Aparicio et al., 2007; Majewska et al., 2006; Uppala et al., 2005; Fukuda et al., 1985; Huovinen et al., 1994; Huovinen et al., 1993; Michna et al., 1992; Reissmann et al., 1992). Mitotic index correlate directly with tumour grade (Romansik et al., 2007). One of the important factors in tumour regression is hormone therapy (Fukuda et al., 1985; Huovinen et al., 1993; Michna et al., 1992). For evaluation of mitotic activity is considered mitotic index, which value is defined as the number of mitotic figures, as well as Ki-67, an immunohistochemical proliferation marker (Yamada et al., 2007; Ninomiya et al., 2006).

DMBA has an effect on cell proliferation and mitotic activity. DMBA induced stimulation of liver cell proliferation has been examined in the small, viviparous fish who are susceptible to induction of liver tumours, and measured by the mitotic index (Schultz et al., 1989). DMBA induces stimulation of the mitotic activity of melanocytes as well as cutaneous hyperpigmentation (Tsambaos et al., 1989). *Iversen et al.* have shown in their study that there is a significant difference between the influences of small and large DMBA doses. Higher DMBA doses will result in a reduction in the DNA synthesis and mitotic activity in the epidermis (Iverson et al., 1988). When DMBA is applied in the solution form, the mitotic activity is at first reduced because of DMBA toxicity (Hassan et al., 1985; Vasilyeva, 1966). When a tumour develops, the rate of cell proliferation, i.e. the cell proliferation index rises (Bagwe et al., 1994; Vasilyeva, 1966). DMBA treatment has been reported to simultaneously accelerate epithelial keratinisation and inhibits epithelial mitotic activity (Fukamachi, 1984).

2.6. Influence and changes in DMBA tumourigenesis

DMBA induced tumourigenesis can be influenced by viral, chemical, hormonal, genetic, immunologic and dietary factors (Blanco-Aparicio et al., 2007). By DMBA administer induction of free radicals are produced and nitrate level is rising (Ozturk et al., 2002). Immobilization stress enhances DMBA induced alteration of liver. Biochemical measurements show that the changes in the levels of marker enzymes in serum were comparable to that of liver tissue (Muqbil et al., 2006). DMBA induces oxidative stress shown by decline in the activities of mitochondrial enzymes that can be inhibited in experiment (Arulkumaran et al., 2007). Cancer is a disease of impaired genome stability. DMBA is a classical multiorgan genotoxic carcinogen. The finding that telomere dysfunction can suppress tumourigenesis has been replicated in mouse DMBA tumour model (Maser et al., 2002). Mutations induced by DMBA in the liver can be found and endogenous ovarian hormones inhibitory effect is examined (Chen et al., 2005). DMBA can induce gene mutations in heart tissue which frequency is studied (Manjanatha et al., 2005). Significant increases in numerical chromosomal aberration, which are commonly used as an indicator of exposure to genotoxic compounds, were detected in DMBA treated rats (Chen et al., 2003; Uppala et al., 2005). For example *Uppala et al* found that genistein has a protective effect on DMBA induced genotoxicity (Uppala et al., 2005). It is suggested that p63 expression may be associated with the regulation of epithelial differentiation and proliferation in DMBA induced carcinogenesis (Chen et al., 2003). Popular are works with chemicals having antitumorigenic properties using DMBA induced tumours (Guo et al., 2006; Sidell et al., 2007).

3. AIMS OF THE PRESENT STUDY

Our interest was to try a new model of topic carcinogenesis using widely used carcinogen DMBA. By tumourigenesis mainly we wanted to pay attention to cell proliferation not inside the tumour, which increase is clear, but in whole system, in several organs. Morphological changes due to topic tumour have been of typical interest in tumour resection line, but not extensively, far from tumour itself.

The aims of the present study:

1. To induce tumours, using a rat model for implanting either DMBA-containing pellets in biological material or uncovered DMBA pellets into abdominal cavity;
2. To study reactive changes in DMBA implants and surrounding tissue;
3. To study the effects of biological tissue on the development of tumours: the time needed for tumourigenesis, tumours dimensions and histological types;
4. To study the activity of cell division and to determine the level of mitotic activity in different intact organs of rats in the process of DMBA induced carcinogenesis.

4. MATERIALS AND METHODS

4.1. Study design

Two different experimental series were carried on, altogether on 150 white rats. Tumours were induced by a cancerogen, DMBA. The reactive DMBA (C18 H10 $(CH3)_2$) was purchased from Fluka AG, Buchs SG in Switzerland. Beeswax, whale's fatty alcohol, activated carbon and a carcinogenic substance were blended, by means of warming, into a mixture. This carcinogenic stuff was made in collaboration with the Institute of Pharmacy of University of Tartu and Peep Veski. Addition these different ingredients were necessary to slow down the release of DMBA and induce tumours more probably. By means of a special instrument with two halves of hollows for filling with mixture, the pellets of a strictly equal diameter (2mm) and mass (3.7mg), each containing 0.038mg of DMBA, were shaped. An experimental model using a foreign material encapsulated in a biological material was applied (Shevtshuk, 1984).

4.1.1. I Group

In the first group 28 animals underwent bilateral adrenalectomy under ether anaesthesia. The removed adrenals were enucleated in vitro by the *Evans* method (Evans, 1936). After the parenchyma had been removed from the organ, the DMBA pellet was inserted into the cavity to be surrounded by the adrenal capsule on all sides, lying in a biological chamber. Then both adrenals were transplanted into the abdominal cavity below the spleen, at the level of the upper apex of the left kidney (Group I).

4.1.2. II Group

Similar DMBA–containing pellets (without biological chamber) were implanted just into abdominal cavity below the spleen, at the level of the upper apex of the left kidney in 72 rats without their adrenal enucleation (Group II).

4.1.3. Control Group

The Control Group consisted of 50 rats. They were operated in a similar manner but the implanted pellets did not contain any DMBA. Also the adrenals of the rats were not enucleated.

4.2. Time

The animals were sacrificed after intervals of 3-4 months, 6-7 months, 9-10 months and 12 months after the implantation of the pellets.

4.3. Measurement of tumours

Three dimensions of the macroscopically diagnosed tumours were measured in centimetres and a scoring system with three levels was developed. The scoring parameter is the summation of tumour three dimensions.

4.4. Histopathological and immunohistchemical investigations

Autopsy tissue samples for histological investigation were taken from the pellet or from the local tumour tissue and with the surrounding tissue, from the small intestine, from the oesophagus, from the liver and from both the glomerular and the fascicular-reticular zones of the adrenal gland.

The tissue samples were fixed in 10% solution of neutral buffered formaldehyde and thereafter routinely processed and embedded in paraffin. Histological sections (5 μm slices) were made and stained with haematoxylin and eosin in routine

manner, with widely used picrofuchsin and haematoxylin after *van Gieson`s* method, or with alcian blue.

To assess the diagnosis of sarcomas the immunohistochemical staining for vimentin and desmin was performed using the streptavidin-biotin complex (Strept ABC/HRP, DAKO Corp, Denmark) indirect staining method. The specific intermediate filament that forms the cellular cytoskeleton was histochemically identified using antibodies (DAKO) to vimentin desmin. A distinctive brown reaction, visible by a light microscope, was developed with DAB (3, 3´ - diaminobenzidine) chromogen.

4.5. Histotopograms

The particles of pellets (foreign material), zones of adrenocortical regeneration, sclerotic connective tissue, granulation tissue and lymphocytic infiltration areas around the pellet were depicted on histotopograms drawn according to a method suggested by *A. Truupõld* (Truupõld, 1973). The fields of vision were scanned horizontally and vertically under a microscope with an ocular network and a preparation shifter (object-lens 8 x 0.20; ocular 7). The results of the observations (the pellet, adrenocortical regenerate, connective tissue, sclerosis, lymphocytic infiltration, granular tissue, degenerations) were drawn on a graph paper where 64 cm^2 corresponds to one field of the ocular network in the sequence they were observed under the microscope. A computerised map with areas wearing differentiating markers was drawn from these histotopograms (Figure 1).

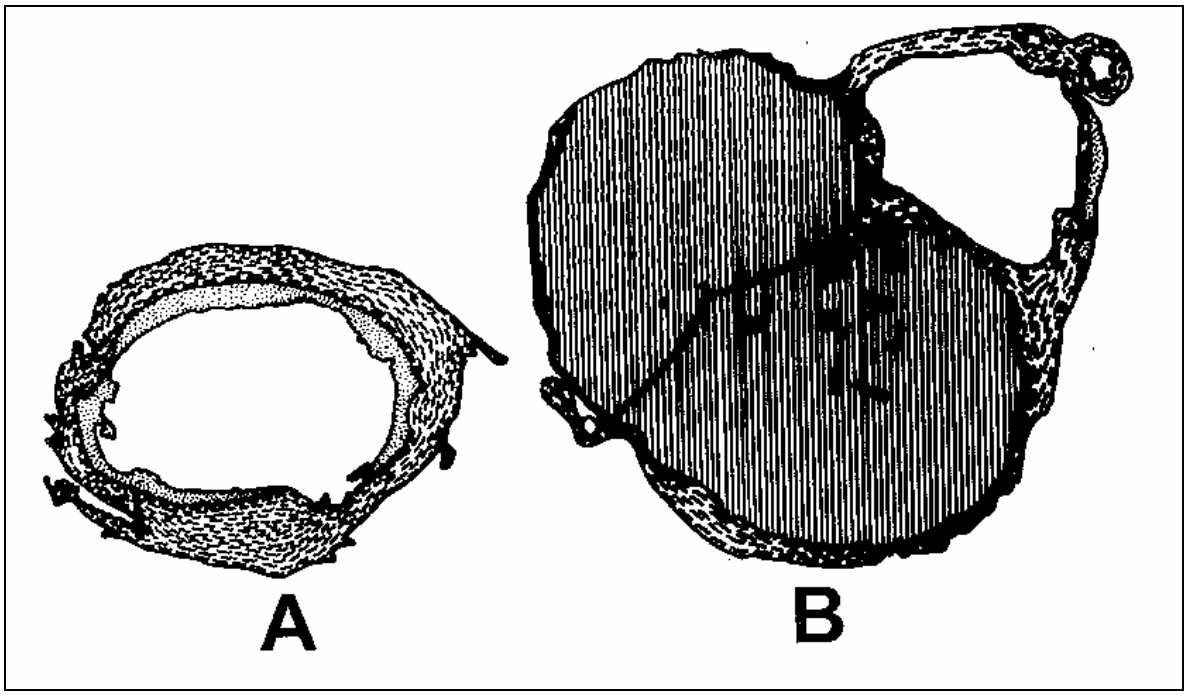


Figure 1. Histotopogram. 2 transplants 3-4 months after the beginning of the experiment (Group I). Labels: white – foreign body; striped – adrenocortical regenerate; wavy – connective tissue; dotted – lymphocytic infiltrate.

The image analysing system Image Pro 3.0 was used to analyse the maps. All fields of the maps were automatically scanned. The computer program read the map and produced the percentage for each object in the squares on the map.

4.6. Statistics

Statgraphics, Microsoft Excel 5.0/7.0, Sigma Statistic program, 2x2 Statistic Tables and Wilcoxon Test were used for statistical evaluation. p signifies the degree of statistical significance and r the presence of correlation.

4.7. Mitotic index

Mitotic activity was determined in the epithelium of the small intestine, in the oesophageal epithelium, in hepatocytes and in the suprarenal glomerular and

fascicular-reticular zones. Proliferation was measured by counting mitotic figures in the histological sections using an ocular square lattice grid and expressed by the mitotic index, i.e. the number of mitosis per 1000 cells in ‰ in the organ. Mitoses were counted under a light microscope in the fields of vision (object-lens 40x0.65; ocular 7). In the small intestine cells and mitoses were counted in 50 crypts, in the adrenal glands in 50 fields of vision, in the liver in 100 fields of vision and in the oesophagus in the cross-section.

Table 1.

Histological types of all tumours by microscopic evaluation after DMBA pellet grafting into abdominal cavity.

Method	n	3-4 months	n	6-7 months	n	9-10 months	n	12 months	n	Total n of tumours
Control Group n = 50	8	-	14	-		6	-	22	-	-	-
I Group n = 28	0/3	-	1/11[a]	*fs	1	4/9	*fs *fs + rms	3 1	4/5	*fs *fs+adc+sc	3 1	9/28[a]
II Group n = 72	0/17	-	17/ 19[a]	*fs *rms *fs+rms+adc *fs+adc *pleos	10 3 1 2 1	10/ 14	*fs *rms *pleos *fs+adc *fs+rms	5 1 2 1 1	15/ 22	*fs *pleos *lipos *fs+adc *fs+adc+rms	10 2 1 1 1	42/72[a]

n – number of tumours / number of rats in group
I Group – DMBA pellets in enucleated adrenals transplanted into abdominal cavity
II Group – DMBA pellets transplanted into abdominal cavity

fs – fibrosarcoma
rms – rhabdomyosarcoma
adc – adenocarcinoma
pleos – pleomorphic cell sarcoma
sc - scirrhus
lipos – liposarcoma

[a] $p < 0.01$

5. RESULTS

5.1. Tumourigenesis

DMBA (in both Groups I and II) induced 51 tumours in 100 experimental white rats. In Group I, where the pellets of DMBA were placed in adrenals as a biological chamber and transplanted into the abdominal cavity, there were nine rats with tumours out of 28. In Group II, where the uncovered DMBA pellet was used, there were 42 rats with tumours out of 72 (Table 1). Thus the tumour prevalence was significantly lower in Group I than in Group II ($p < 0.05$).

The first tumours were diagnosed 6-7 months after grafting the DMBA pellets. At that time in Group I the number of rats with tumours was significantly lower ($p < 0.01$). There were found no difference in number of tumours between the groups sacrificed at 9-10 and 12 months later (Table 1). The size of the tumours was varied – from 0.5 cm in diameter to 8x7x4 cm, noticed in the ninth month from the beginning of the experiment (Table 2).

Table 2. Macroscopic evaluations of tumourigenesis in three dimensions after DMBA pellet grafting into abdominal cavity (in cm). (* one examinee, having 1-3 tumour foci)

Method	n	3-4 months	n	6-8 months	n	9-10 months	n	12 months
Control	8		14		6		22	
I Group	0/3	-	0/6	-	4/8	*2x2x1 *8x4,5x5 *0,5x0,5x0,5; 0,5x0,5x0,5; 0,5x0,5x0,5 *3x2,5x2,5; 4x2,5x2,5	4/5	*1x1x1 *d 0,5 *d 0,5 *7x5x5; 5x5x5; d 0,5
II Group	0/17	-	3/19	*0,5x1x1 *2x2x2 *9x3x3	3/14	*2x2x2 *7x8x4 *4x4x6	8/22	*6x5x2 *1,5x0,5x0,5 *0,5x0,5x0,5 *0,5x0,5x0,5 *1,5x0,5x0,5 *1,5x2x1 *3x3,5x1,5 *1x1x1

The largest dimension was 9 cm in length. Some examinees have arisen two to three tumour foci - the growth was multicentric. Figure 2 shows all macroscopically detectable tumours presented by their largest dimensions.

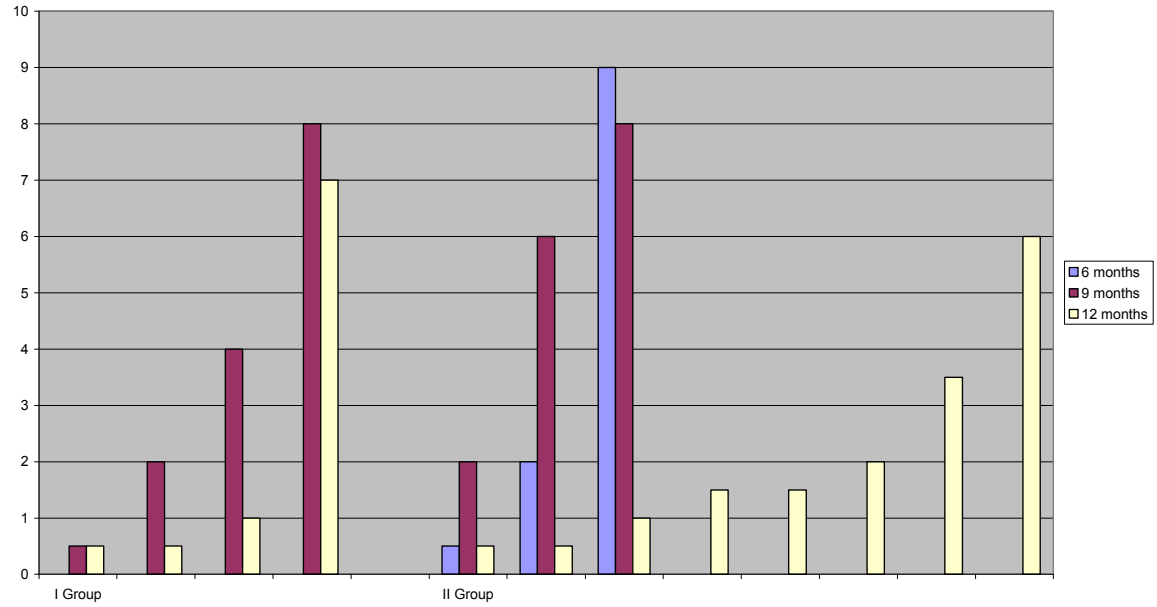
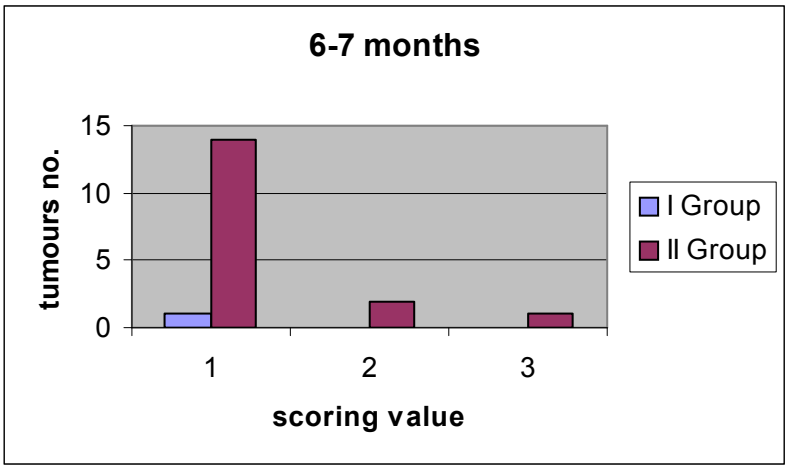
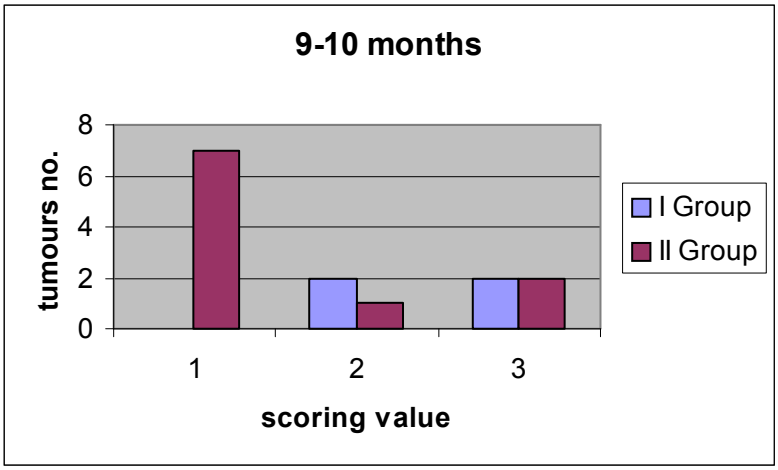


Figure 2. Macroscopic evaluations of tumourigenesis (by the largest dimension in cm)

For scoring, all three tumour dimensions were united and these, of several foci, also. We mentioned tumours in three levels. 1 – only microscopically diagnosed tumours; 2 – (up to 12 cm) macroscopic tumours of medium size; 3 – (13 - 33.5 cm) large tumours. According to the scoring results of tumour size (Figure 3) the tumourigenesis was significantly lower in the Group I, than in Group II ($p < 0.01$) after 6-7 months. After 9-10 and 12 months of the experiment Groups I and II did not display any difference in the tumour size.

The tumours were nodular, of dense consistency, the surface of cut slices were whitish–greyish-pinkish with brownish and reddish nidi, in places with necrotic nidi and haemorrhage and even vacuoles filled with fluid or pus. The tumours were arisen locally with their localisation in the gut. Some larger tumours had spread into the peritoneal and retroperitoneal cavities as well as into the thoracic cavity, coalescing with spleen, intestine, left kidney and mesentery. Microscopically the tumours were of different histological type (Table 1). In most cases the parenchyma consisted of fibroblas-like spindly cells and collagen fibres, the





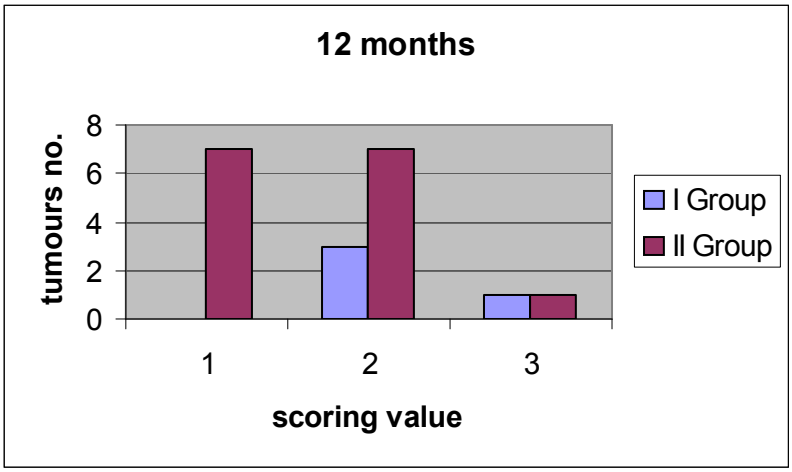


Figure 3. DMBA induced tumours size scoring, after different time.

1 – microscopically diagnosed tumours; 2 – medium size tumours; 3 – large tumours.

amount of which was different in different parts of the tumour. The cells and fibre fascicles were intersected and they were of different shape and size, the nuclei were hyperchromic with thick chromatin lumps. Atypical mitoses were evident. Between the cells there were collagen fibres which formed fascicles of different thickness. Between the fascicles there were irregular groups of infiltrated round cells. Blood vessels and perivascular connective tissue formed stroma. On the basis of the characteristic histological structure, the diagnosis of fibrosarcoma could be given. The main type was fibrosarcoma in both group, of 51 tumours 41 fibrosarcomas were found (Figure 4A). There were also big elongated multinuclear myosymplast-like atypical formations present. Their cytoplasm was vacuolated in places and in places homogenous. Fascicles of myofibrils could be observed in myosymplasts. The nuclei were of different shape and hyperchromatic. Histological changes suggested rhabdomyosarcoma. There were eight rhabdomyosarcomas present (Figure 4B). Histochemical reactions with anti-vimentin and anti-desmin antibodies confirmed the diagnoses of fibrosarcoma and rhabdomyosarcoma in our study. There was a specific intermediate filament forming the cellular cytoskeleton. Antibodies reacted strongly with vimentin or desmin and labelled cells of mesenchymal and muscular origin. DAB chromogen produced an insoluble brown product that stains the nuclei of corresponding tumour cells browny (Figure 5).

Several combined tumours were diagnosed: fibrosarcoma with rhabdomyosarcoma, adenocarcinoma (Figure 4C), scirrhus (Figure 3D). In one case there was found liposarcoma (Figure 4E). One more anaplastic tumour type was present in four cases - pleomorphic cell sarcoma (Figure 4F). In Group II there were more different tumour types singly or in combinations, than in Group I (Table 1).

Figure 4A. Fibrosarcoma. In majority the parenchyma is made up by fibroblast-like spindly cells and collagen fibres, the amount of which is different in different parts of tumour. The cells and fibres fascicles are intersected in different shape and size, the nuclei are hyperchromic, with thick cromatin lumps. Between the cells there are collagen fibres which formes fascicles of different thickness. Blood vessels and perivascular connective tissue with irregular groups of round cells infiltrate as stroma (Haematoxylin and picrofuchsin, magnification 40x0.85).

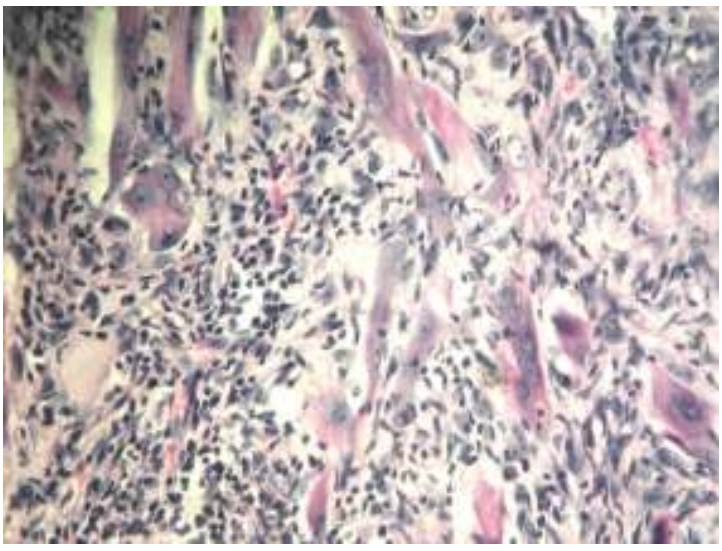


Figure 4B. Rhabdomyosarcoma. Big elongated multinuclear myosymplast-like atypical formations are in evidence. The cytoplasm is vacuolated in places and in places homogenous. In myosymplasts fascicles of myofibrils can be observed. The nuclei are of different shape, hyperchromic (Haematoxylin and eosin, magnification 40x0.85).

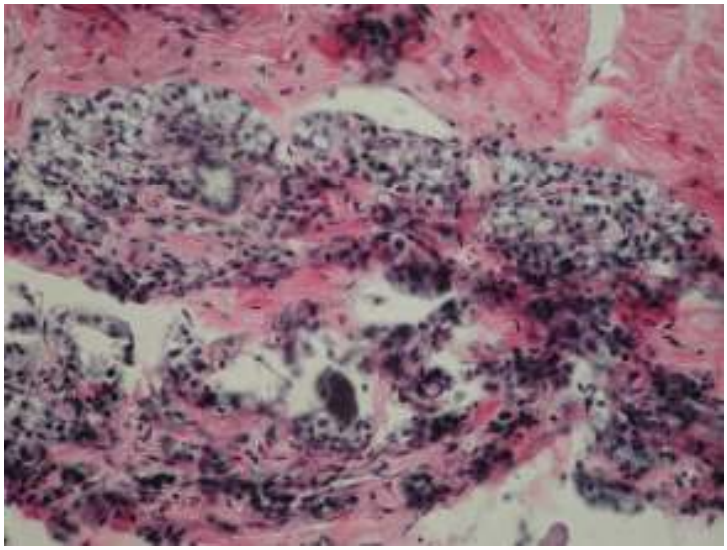


Figure 4C. Adenocarcinoma. Atypical gland form formations can be observed. Polymorphous cells of epithelial origin with light-coloured nuclei are located in groups (Haematoxylin and eosin, magnification 40x0.85).

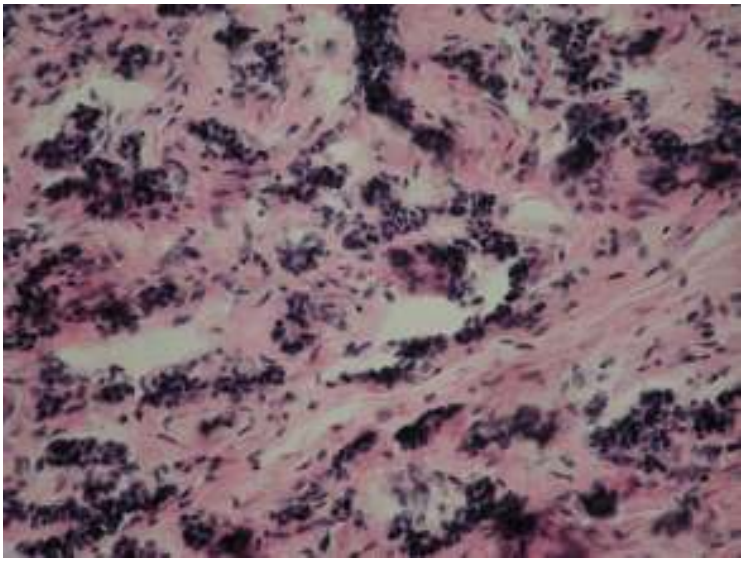


Figure 4D. Scirrhus. Less differentiated small dark cells in groups between which there are plenty of connective tissue (Haematoxylin and eosin, magnification 40x0.85).

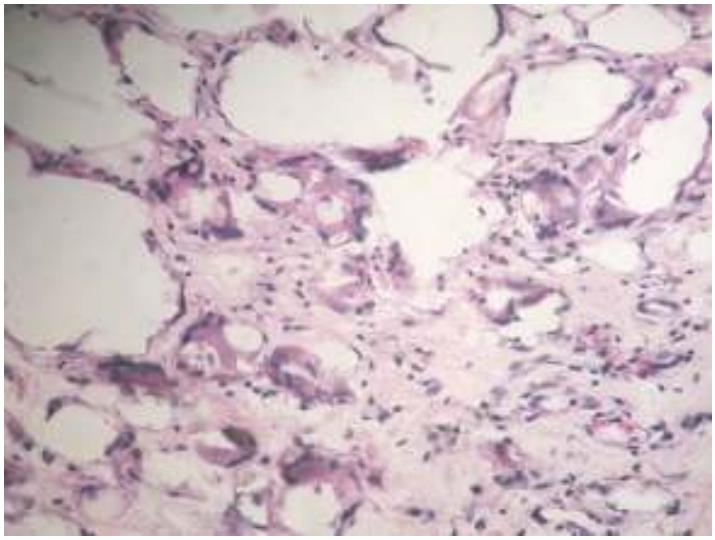


Figure 4E. Liposarcoma. Tissue resembles fatty tissue with lipocytes, but has plenty of atypical cells (Haematoxylin and eosin, magnification 40x0.85).

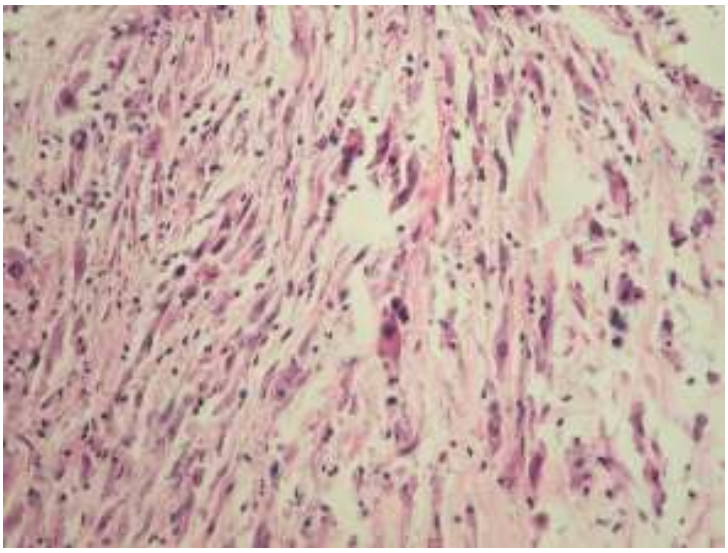


Figure 4F. Pleomorphic (polymorphic) cell sarcoma. Anaplastic sarcoma with cells of very different shape and size with hyperchromic nuclei of different chromatin containing is seen (Haematoxylin and eosin, magnification 40x0.85).

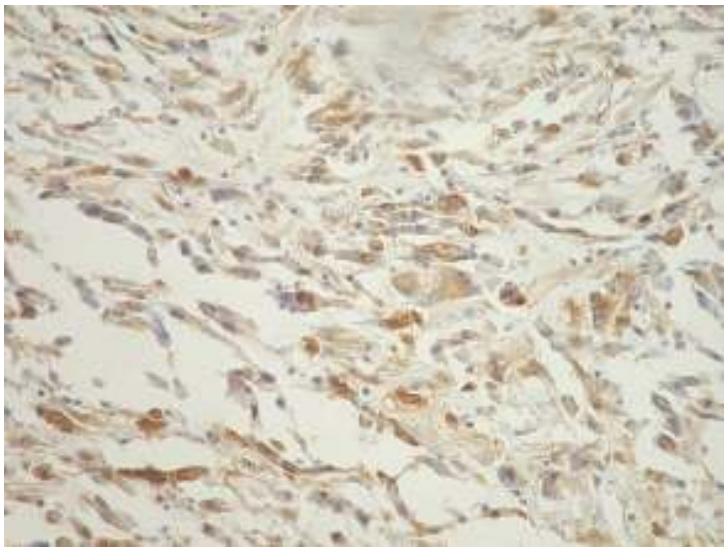


Figure 5. Pleomorphic (polymorphic) cell sarcoma (Vimentin, magnification 40x0.85).

5.2. Reactive changes around the implants

The pellets were either intact or were divided into segments by connective tissue. The foreign bodies presented microscopically themselves as structureles black mass, yet the pellet could not be found in very large tumours. Usually the areas containing foreign bodies were encapsulated by the connective tissue (Figure 6).

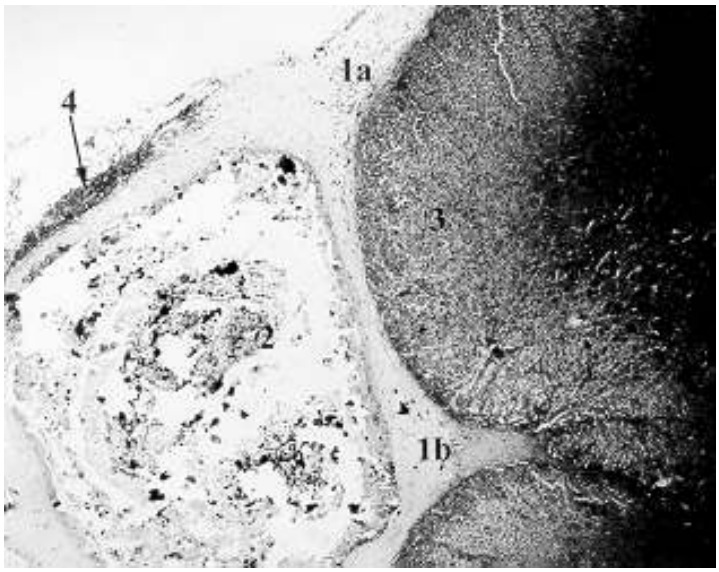


Figure 6. Implant region. 1 - connective tissue surrounds foreign material and adrenocortical regenerate, 1a - granular tissue, 1b - hyalinosis; 2 - foreign material

(pellet); 3 - adrenocortical regenerate; 4 - lymphocytes (Haematoxylin and eosin, magnification 10x0.20).

Collagen fibres and their fascicles surrounded the foreign material concentrically. In some areas polyblasts, big and light epithelioid cells and fibroblasts with lymphocytes could be observed, confirming young connective tissue, e.g. granular tissue. In other places hyalinosis could be seen. Atypical cells – the beginning of tumours – could be noticed around the foreign bodies, next to or inside the connective tissue. In Control Group and Group II the picture of pellet organisation was similar.

In Group I the connective tissue was surrounding the adrenocortical regenerate. The adrenocortical regeneration formed nodes, the differentiation of proliferated cells into a glomerular zone and a fasciculat–reticular zone was observed. In structure of the adrenocortical regenerate, there are plenty of dilated capillaries and haemorrhages. Some cases light cells with vague contours can be seen in the middle of the adrenocortical tissue mass – an amorphous area of small granules. Here we have to do with dystrophic and necrobiotic changes in the adrenal regenerate under conditions of functional overstrain, a phenomena decribed by *A. Truupõld* in his writings (Truupõld, 1969; Truupõld 1976)

Organisation, i.e. proliferation of connective tissue was more markedly present in the experimental groups than in the control group. The reactive changes in the Experimental Groups and the Control Group are presented in per cent in Table 3. Lymphocytic infiltration around the pellet in all periods was statistically higher in Group I compared with Control Group ($p < 0.01$). Particularly, in Group I the lymphocytic infiltration rate in the rats was remarkably lower in case of arising tumours (4.07, 0 and 5.08%) than without it (8.79, 8.24 and 18.16%) (Table 3).

Table 3. Reactive changes manifestation (%) around implants (according to histotopograms). ($p < 0.05$ Control Group versus Group I).

		Median / Range			
Time	Group	Granular tissue		Lymphocytic infiltration	
		Without tu	With tumour	Without tu	With tumour
3-4 months	Control I II	1.00/0-5.69 0 12.09/0-36.74		0* 7.97/2.71-13.24 3.45/0-20.79	
6-7 months	Control I II	1.64/0-13.36 0 6.75/0-13.5	 30.47/0-60.95 3.03/0-12.85	1.25/0-8.41* **8.79**/0-23,6 5.05/2.34-7.77	 **4.07**/0-8.15 2.79/0-10.63
9-10 months	Control I II	3.08/0-15.4 0 3.32/0-9.96	 0 8.13/0-57.85	0.26/0-1.29* **8.24**/1.95-13.19 4.57/0-9.45	 **0** 1.52/0-6.64
12 months	Control I II	0.42/0-5.03 0 16.66/0-61.78	 0 1.75/0-6.66	0* **18.16**/18.16 4.61/0-10.55	 **5.08**/0-10.16 3.24/0-19.17

5.3. Mitotic activity

Data expressing the level of mitotic activity in different organs both in the Experimental Group (Group II) and the Control Groups expressed by the mitotic index (MI) are given in ‰ in Table 4.

MI in the small intestine in the third month from the beginning of the experiment was 81.2‰. In comparison with the Control Group (MI = 74.1‰) the statistical difference was not significant ($p > 0.05$). But in the sixth and ninth months a significant rise in the mitotic activity in the epithelial cells could be observed, the MI values being 147.0‰ and 119.3‰ respectively ($p < 0.001$). By the twelfth month the mitotic activity had dropped back to the level of the Control Group and even lower – the MI was 56.6‰. The difference between the Control Group and the Experimental Group was statistically significant ($p < 0.01$). Figure 7A presents the range of changes.

No statistically significant changes took place in the oesophageal epithelium. There was a small decrease present in the Experimental and the Control Groups, jet it was not statistically significant ($p > 0.05$) (Fig. 7B).

Table 4. Mitotic index ($^{o}/_{oo}$) in different organs of rats in different experimental time (Group II).

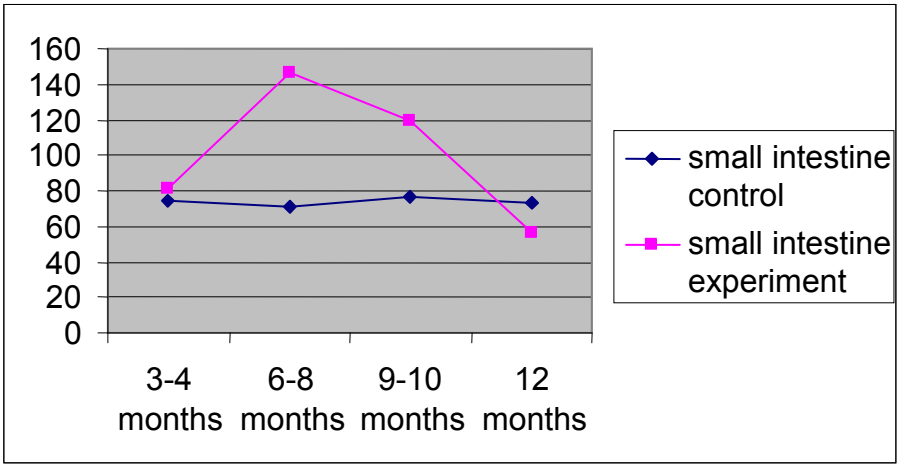
		3 months	*6 months*	*9 months*	*12 months*	*total*
Number of rats	**control**	**8**	**8**	**6**	**18**	40
	experiment	**17**	**19**	**14**	**22**	72
	*with tumour		17	10	15	
	*without tumour		2	4	7	
Small intestine	control	74.1+-8.2	70.9+-4.7	77.1+-7.2	72.9+-4.9	
	experiment	81.2+-6.6	147.0+-3.2	119.3+-4.6	56.6+-3.2	
		p>0.05	p<0.001	p<0.001	p<0.01	
	*with tumour		146.7	122.8	54.6	
	*without tumour		149.5	112.1	60.7	
Esophagus	control	17.1+-1.3	13.8+-1.3	13.8+-2.6	11.8+-1.6	
	experiment	14.3+-1.2	12.0+-1.2	11.9+-1.9	9.9+-1.0	
		p>0.05	p>0.05	p>0.005	p>0.05	
	*with tumour		12.4	12.6	8.4	
	*without tumour		8.5	10.3	13.1	
Liver	control	1.3+-0.3	1.2+-0.2	1.5+-0.2	1.3+-0.2	
	experiment	1.4+-0.2	3.9+-0.4	4.3+-0.2	4.6+-0.4	
		p>0.05	p<0.001	p<0.001	p<0.001	
	*with tumour		4	4.6	5.4	
	*without tumour		2.7	3.7	2.6	
					p<0.001	
Glomerular zone	control	0.25+-0.06	0.20+-0.07	0.25+-0.02	0.22+-0.01	
	experiment	0.64+-0.09	0.24+-0.05	0.45+-0.16	0.45+-0.06	
		p<0.05	p>0.05	p>0.05	p<0.01	
	*with tumour		0.25	0.47	0.49	
	*without tumour		0.1	0.39	0.37	
Fascicular-reticular zone	control	0.46+-0.11	0.45+-0.05	0.47+-0.04	0.46+-0.03	
	experiment	0.64+-0.06	0.45+-0.05	0.47+-0.08	0.91+-0.19	
		p>0.05	p>0.05	p>0.05	p<0.05	
	*with tumour		0.46	0.43	1.2	
	*without tumour		0.39	0.57	0.28	
					p<0.05	

*with tumour - MI in the Experimental Group with tumour development *without tumour - MI in the Experimental Group without tumour development

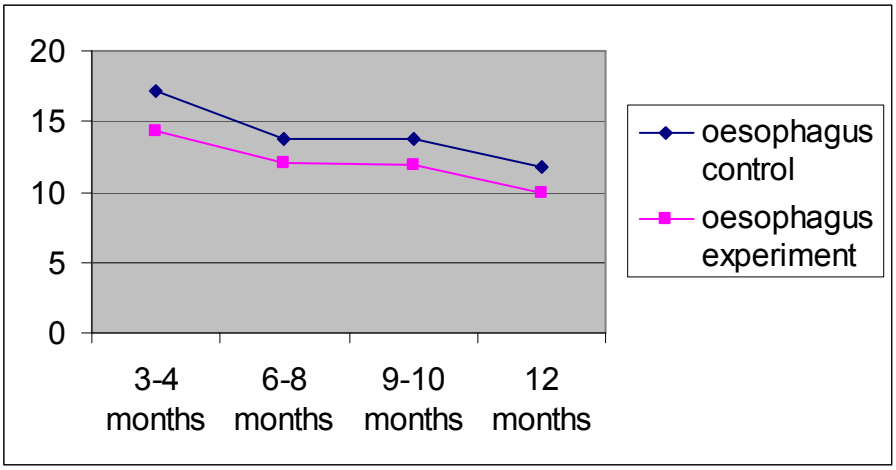
After 3 months from the beginning of the experiment, mitotic activity of hepatocytes in the Experimental Group (MI = 1.4‰) was close to that in the Control Group (MI = 1.3‰) ($p > 0.05$), but afterwards, it began to rise remaining on a higher level than in the Control Group (MI = 3.9-4.6‰, $p < 0.001$) (Fig. 7C).

The changes took place differently in the zones of the adrenal gland. In the glomerular zone, a significant rise occurred in the third month of the experiment. The MI in the Experimental Group was 0.64‰, and 0.25‰ in the Control Group ($p < 0.05$). Then the mitotic activity of the Experimental Group decreased to the level of the Control Group; another rise took place in the twelfth month of the experiment (Fig. 7D). In the fascicular-reticular zone a significant rise in the MI value occurred only in the twelfth month of the experiment – the MI was 0.91‰ in the Experimental Group and 0.46‰ in the Control Group ($p < 0.05$) (Fig. 7E).

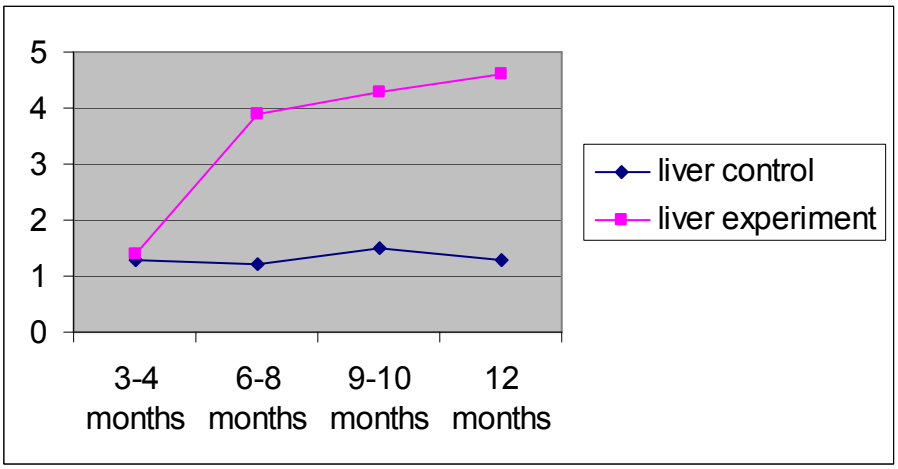
We also studied the correlation of mitotic activity. The mitotic index of different organs shows single cases of both positive and negative correlations between the MI values of the small intestine and of the other organs: so in the third month of the experiment the correlation of MI(small intestine) and MI(liver) $r = 0.609$, $p < 0.01$; MI(small intestine) and MI(glomerular zone of the adrenal gland) $r = -0.588$, $p < 0.05$; in the sixth month of the experiment the correlation of MI(small intestine) and MI(liver) $r = -0.532$, $p < 0.05$; in the twelfth month of the experiment the



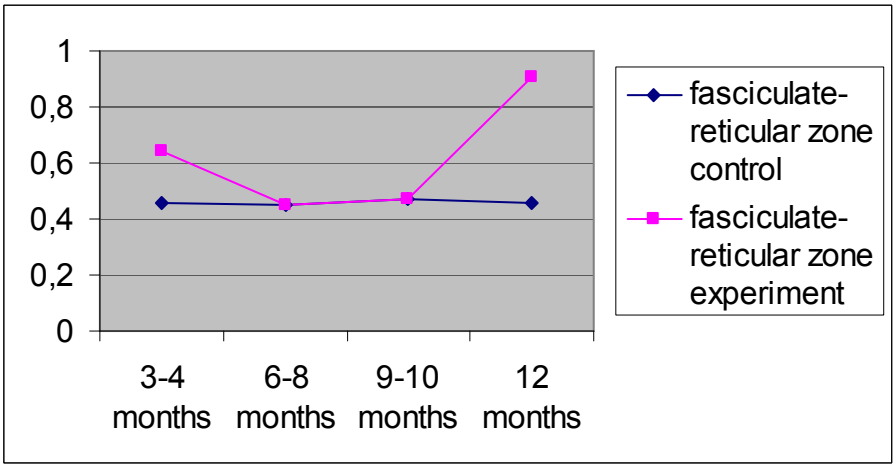
7A


20
15
10
5
0
3-4 months
6-8 months
9-10 months
12 months
oesophagus control
oesophagus experiment

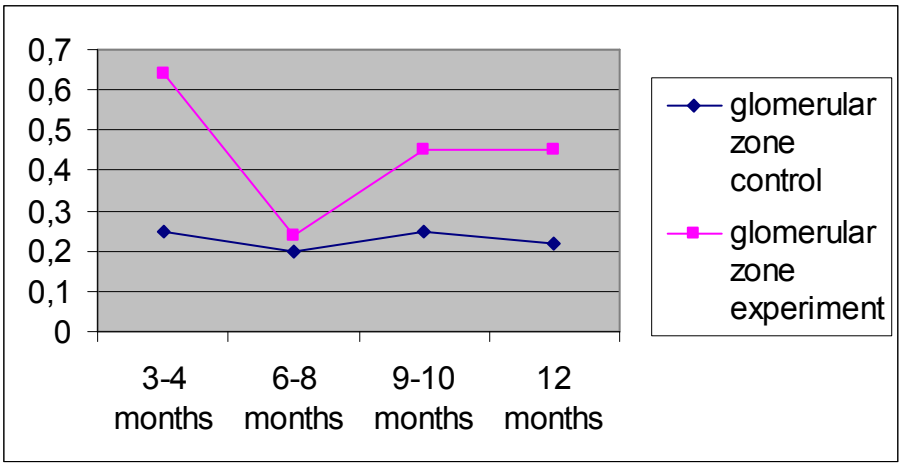
7B


5
4
3
2
1
0
3-4 months
6-8 months
9-10 months
12 months
liver control
liver experiment

7C


1
0,8
0,6
0,4
0,2
0
3-4 months
6-8 months
9-10 months
12 months
fasciculate-reticular zone control
fasciculate-reticular zone experiment

7D



7E

Figure 7. Mitotic index per 1000 cells in different organs during a one year – experiment of DMBA pellet grafting into the abdominal cavity

correlation of MI(small intestine) and MI(fascicular-reticular zone of the adrenal gland) $r = -0.529$, $p < 0.05$.

The mitotic activity in rats with and without tumours in the Experimental Group was also compared. The only differences occurred in the correlation between the values of the liver and the fascicular-reticular zone of the adrenal gland in the twelfth month of the experiment. The MI value for hepatocytes in rats with tumour was higher than the corresponding value in rats without tumour – 5.4‰ and 2.6‰ respectively ($p < 0.001$), in comparing the values for MI in the fascicular-reticular zone the picture was about the same: in rats with tumour the MI was 1.2‰, in rats without tumour – 0.28‰ ($p < 0.05$).

6. DISCUSSION

6.1. Tumourigenesis

We found that 0.038 g of DMBA could induce the formation of tumour which developed around the DMBA pellets in a period of 6-7 months from the start of the experiment. Our experiment induced mesenchymal and epithelial types of tumours, sometimes occurring together in one and the same experimental animal. The most frequent histological form encountered in our experiment was fibrosarcoma. This is in accordance with previous studies, as tumours developing after DMBA implantation, originate either from the epithelium (Nishida et al., 1998; Shiba et al., 1982; Soloven et al., 1963) or from the mesenchyma (Fathy, 1993; Whitmire et al., 1978). Our induced tumours growths were multi-nodular suitably with *Ninomiya et al.* description to DMBA induced tumours (Ninoma et al., 2006). Intrasplenic DMBA grafting with biological material (ovarian tissue) has been carried out by *Nishida, T. et al.* and observed two different histological types of tumours from which one was adenocarcinoma (Nishida et al., 1998). The grafting a DMBA pellet under the edge of spleen in a biological chamber (adrenal) or uncovered induced the same types of local tumours.

6.2. Reactive changes

Organisation process in its different stages from granulation tissue to hyalinosis as well as lymphocytic inflammation could be observed in the study. The lymphocytic infiltration due to a foreign body was lower by arising tumours. Inflammatory reaction due to a foreign body inhibits the development of a tumour and the tumour in its turn reduced the inflammatory reaction induced by the foreign body (Ruggiero et al., 1989). The idea that there is a relationship between inflammatory processes and the development of cancer is as old as its elusive. There exits a number of possible mechanisms, e.g. macrophages are also thought to influence cancer development in addition to their role in the generation of active oxygen

radicals (Heppner et al., 1988). DMBA has the ability of inducing inflammation (Brandes et al., 1991).

6.3. The effect of biological tissue

In Group I there was less connective tissue and sclerosis. Seemingly the regenerating biological material slows down the process of organisation of foreign material and thus tumourigenesis is also slower. In Group I tumourigenesis developed more slowly, only 5.6% of tumours formed in Group I and 94.4% in Group II in the 6-7 months of the experiment. Most of the tumours started later than 9 months after the implantation.

We compared two rat models of tumourigenesis and found that grafting a DMBA pellet under the edge of spleen in a biological chamber (adrenal) or uncovered, induced the same types of local tumours, however the number of tumours was significantly lower with biological material. Biological material with its reactions slows down DMBA-induced local tumours. This finding could be explained with the stronger immune reaction, induced by biological material.

6.4. Mitotic activity

We found that DMBA induces the changes in mitotic activity. From other studies we had learned that DMBA was able to induce local changes in mitotic activity. Some authors found MI increases (Bagwe et al., 1994; Bednarek et al., 1997; Kamenswaren et al., 1976; Lavi et al., 1982; Niskanen, 1962; Ramchandani et al., 1998; Schultz et al., 1989; Tsambao et al., 1989), some authors found mitotic activity decreases (Fukamachi, 1984; Hassan et al., 1985). In our work we have mentioned that different organs react differently to DMBA presence in the organism. A persistent rise in mitotic activity was observed in the liver. *Iversen et al.* have reported that higher doses of DMBA would lead to an initial reduction in mitotic activity (Iversen et al., 1988). At first the decrease is due to the toxicity of DMBA and damage caused by DMBA and later an increase in mitotic activity takes

place (Fukamach, 1984; Hassan et al., 1985; Vasilyeva, 1966). *Shultz* et al. found that the mitotic index was the highest on the final days of the experiment and some doses could even be lethal to the viviparous fish, but sub lethal doses would increase mitotic activity (Schultz et al., 1989). 0.038 mg of DMBA did not cause visible intoxication in rats. The high MI values in the liver may be due to detoxication process. The data demonstrate that all the mitotic activity changes occurred locally. But our investigation is the first to suggest that the DMBA effect on cell proliferation can be wider as we could observe different changes in mitotic activity take place in intact organs far from the place of DMBA treatment.

Independent changes took place in the organs. There was no general correlation between the MI values of different organs. MI values were lower in the first months of experiment. Stress by inhibition of mitotic activity can explain the finding and higher mitotic indexes can be seen when the organs began to recover from the stress (Sylvester et al., 1982). It is also possible that the amount of active DMBA may be small, as the DMBA release from carbon pellets is slow. No cell proliferation was observed in the oesophagus after DMBA implantation. But MI values changed in the small intestine, the liver and the adrenal glands. The MI values in the small intestine rise for a long time, but finally cell proliferation is inhibited. *Pozharisski* found an increase in the mitotic index in the large intestine in the third month after the systemic application (injections) of DMBA (Pozharisski, 1976). Similar MI increases in the intestine have been found after injections of other carcinogenic agents (Altmann et al., 1984). The MI values in the liver rose and then continued high. Changes in MI values did not depend on whether tumours developed or not after DMBA treatment, the only exceptions being the liver and the fascicular-reticular zone of the adrenal gland which had higher MI values in rats with tumours. MI values did not depend on whether the tumour was macroscopic or microscopic, i.e. they did not depend on the size of the tumour.

The results of this study suggest that DMBA promotes systemic cell proliferation regardless the magnitude and kind of reactive changes to DMBA, or tumours that may develop due to DMBA influence.

7. CONCLUSIONS

1. DMBA induces both mesenhymal and epithelial tumours
2. The lymphocytic infiltration due to a foreign body is lower by arising tumours
3. Adrenal regeneration slows down the organisation of foreign material
4. Biological material (adrenals) around carcinogen stuff protect from tumour
5. DMBA promotes systemic cell proliferation i.e. not only locally, but also in different intact organs.

This work may have importance among several anti carcinoma genesis findings. Adrenals (biological material) protect from tumour. This finding could be explained with the stronger immune reaction, induced by biological material but this may be associated with their hormones too which require further examination.
We also pay attention to cell proliferation by cancer genesis not topically, as from literature found, but extensively and changes are found not only inside as well as around the tumour but widely.

8. SUMMARY IN ESTONIAN

NEERUPEALISED KANTSEROGEENI
SISALDAVATE IMPLANTAATIDE ÜMBER PIDURDAVAD KASVAJA TEKET

Kantserogeneesiprotsessi ja seda mõjutavate tegurite uurimine on aktuaalne kogu maailmas. Mitmeti indutseeritud kasvajamudelite erinevate mõjutamisviisidega püütakse leida kasvaja teket inhibeerivaid võimalusi. Kuna kasvaja on rakkude pidurdamatu vohang mitooside jadaga, siis kasvaja teket ja progressi saab jälgida ka mitootilise aktiivsuse uurimise abil. Rakkude proliferatsiooni näitajaks kasutatakse mitootilise aktiivsuse määramist, mida väljendatakse mitootilise indeksi näol. Kantserogeeni manustamisel on ilmne kasvaja teke ja mitootilise aktiivsuse tõus vastavas piirkonnas. Kuidas aga reageerib organism üldiselt kantserogeeni ja kasvaja olemasolu korral? Kirjandusest on näha, et uuritakse kasvajat ennast ja ümbritsevat piirkonda kuni resektsioonijooneni, kuid kas on potentsiaalne võimalus mitootilise aktiivsuse tõusuks ka eemal asetsevates intaktsetes elundites?

DMBA (7,12-dimetüülbensantratseen) on sünteetiline polütsükliline aromaatne süsivesinik, mida laialt kasutatakse eksperimentaalses meditsiinis ja bioloogias kantserogeneesiprotsessi uurimisel kasvajate indutseerimiseks. DMBA tekitab kasvajat manustades nii lokaalselt kui ka söögiga suu kaudu või intravenoossete süstidega manustades. On teada, et otsene DMBA kontakt koega võib tekitada tugevaid alteratiivseid muutusi tänu toksilisusele. DMBA aeglase vabanemise korral kudedesse on aga kasvaja tekke protsent kõrge.

Kasvaja indutseerimisel pakkus meile huvi, kuidas mõjutab kasvaja teket bioloogiline materjal, mis eksperimendis sisestada organismi koos DMBA-ga. Kirjandusest analoogseid võrdlusandmeid ei leidnud. Sellest lähtudes sai kasutatud uudset kantserogeneesimudelit.

Konkreetsed ülesanded

1. Indutseerida kasvaja, kasutades uudset rotimudelit, implanteerides DMBA sisaldusega vahakuulikesed kõhuõõnde kas ümbritsetuna bioloogilisest materjalist (neerupealisest) või ilma selleta.
2. Uurida reaktiivseid muutusi DMBA implantaatides ja nende ümbruses.
3. Uurida bioloogilise materjali mõju kasvaja tekkele, pöörates tähelepanu kasvaja tekke kiirusele, kasvaja mõõtmetele ja histoloogilistele tüüpidele.
4. Uurida rakkude proliferatiivset aktiivsust erinevates intaktsetes elundites DMBA toimel tekkiva kasvaja olemasolul roti organismis.

Materjal ja metoodika

Eksperiment viidi läbi 150 valgel rotil. Moodustati 2 katsegruppi ja üks kontrollgrupp. Soojendades valmistati segu mesilasvahast, vaala rasvalkoholist, söest ja DMBA-st. Vastavaid aineid sai kasutatud, et kantserogeeni vabanemine oleks aeglasem ja kasvajad tekiksid suurema tõenäosusega. Spetsiaalse instrumendiga vormiti kantserogeenimassist spetsiaalsed kuulikesed diameetriga 2 mm, massiga 3,7 mg ning DMBA sisaldusega 0,038 mg. Esimese grupi moodustasid 28 rotti, kellel eeternarkoosis viidi läbi bilateraalne adrenalektoomia. Neerupealised enukleeriti ja eemaldatud parenhüümi asemele asetati DMBA kuulike, ümbritsetuna neerupealisekapslist. Mõlemad neerupealised transplanteeriti kõhuõõnde põrna serva alla. Analoogsed DMBA kuulikesed ilma ümbritseva bioloogilise materjalita ja ilma eelneva adrenalektoomiata implanteeriti 72 rotil samuti kõhuõõne samasse piirkonda, moodustades teise katsegrupi. Kontrollrühmaks olid samasuguselt opereeritud, kuid DMBA-d mittesisaldavate kuulikestega 50 rotti. Loomad hukati vastavalt 3-4 kuud, 6-7 kuud, 9-10 kuud ja 12 kuud pärast kuulikese implanteerimist. Koetükid võeti organiseeruvast kuulikesest ja teda ümbritsevast koest, tekkinud kasvajakoldest, peensoolest, söögitorust, maksast ja neerupealistest. Koetükid fikseeriti 10% neutraalses formaliinilahuses, sisestati rutiinmeetodil parafiini ja nendest valmistati histoloogilised preparaadid. Koelõigud värviti hematoksüliini ja eosiiniga, pikrofuksiini ja hematoksüliiniga van Giesoni järgi ning altsiaansinisega. Immuunhistokeemia meetod oli vajalik kasvajate diagnostikas, kasutades markeritena tsütokeratiini, vimentiini ja

desmiini. Võõrkehast (DMBA kuulike), regenereeruvatest neerupealistest, lümfotsütaarsest infiltraadist ja sidekoest joonistati histotopogrammid, mida hiljem analüüsiti Image Pro 3.0 programmiga ja toodi välja vastavate piiralade protsendiline jaotus. Statistiliseks töötluseks kasutati programme Statgraphics, Microsoft Excel 5.0/7.0 ja Sigma Statistic, 2x2 statistilisi tabeleid ning Wilcoxon Testi. Mitootiline indeks määrati peensooles, söögitorus, hepatotsüütides ja neerupealise glomerulaar- ja fastsikulaat-retikulaartsoonis. Mitootiline aktiivsus väljendati mitootilise indeksina, s.o. mitooside hulgana 1000 raku kohta elundis.

Töö tulemused ja järeldused

1. Eksperimentaalselt tekkisid nii mesenhümaalsed kui epiteliaalsed tuumorid, mis esinesid kas eraldiseisvatena või ka kombineeritult ühel ja samal katseloomal. Kõige sagedasem tekkinud histoloogiline kasvajavorm oli fibrosarkoom. Makroskoopiliselt olid kasvajad sõlmelised ja korduvalt esines rotil mitu eraldi sõlme – tegemist oli multitsentrilise tekkega kasvajaga.
2. Võõrmaterjali (kuulikest) oli näha erinevates organisatsiooniprotsessi staadiumides granulatsioonkoest hüalinoosini välja. Kasvaja tekke korral täheldasime väiksemat lümfotsütaarse infiltratsiooni reaktsiooni, kui see oli katsegrupi rottidel, kellel ei tekkinud kasvajaid. Võõrkehast tingitud põletikuline reaktsioon inhibeerib tuumori teket ja kasvaja ise langetab võõrkeha poolt tekitatud lümfotsütaarset reaktsiooni.
3. I katsegrupis oli leida vähem sidekoelist reaktsiooni. Neerupealiste regeneratsioon surub maha võõrkeha organisatsiooni.
4. Kahe katsegrupi võrdlusel selgus, et DMBA kuulike kõhuõõnes bioloogilises kambris tekitas vähem kasvajaid, kui vastav DMBA kuulike ilma neerupealisest ümbrisetuna. Neerupealised, ümbritsedes kantserogeenisisaldusega implantaati, takistavad kasvaja teket.
5. DMBA mõjutab mitootilist aktiivsust mitte ainult lokaalselt, vaid süsteemselt terves organismis. Erinevad intaktsed organid reageerivad erinevalt.

Töö omab tähtsust eksperimentaalse kantserogeneesi uuringute vallas. Leidsime, et neerupealised ümber kantserogeense aine vähendavad eksperimendis kasvaja

teket. Kasvaja tekke pidurdumine võib olla seotud bioloogilisest materjalist tingitud immuunreaktsiooni tugevnemisega. Edasist uuringut vajaks, kas tegu on lihtsalt bioloogilise materjali mõjuga või ka neerupealise hormonaalse antitumorogeneesse toimega .

Samuti pöörasime tähelepanu rakkude proliferatsioonile kantserogeneesi protsessis, kuid mitte lokaalselt, mille kohta võib kirjanduses piisavalt viiteid leida, vaid just üldiselt, seega erinevates intaktsetes elundites. Leidsime, et mõju on kogu organismile, kuigi erinevates elundites veidi erinev.

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PUBLICATIONS

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Mesila I.

Biological material around DMBA implants reduces tumorigenesis.

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BIOLOGICAL MATERIAL AROUND DMBA IMPLANTS REDUCES TUMORIGENESIS

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ABSTRACT

DMBA-containing pellets were implanted below the spleen into the abdominal cavity of rats to give rise to local epithelial and mesenchymal malignant tumours. Two types of DMBA-containing intraabdominal implants were compared — DMBA-containing pellets surrounded by biological material (adrenal) and uncovered DMBA pellets. The time of formation of tumours, their size and histological type, reactive changes in the pellet and around it were recorded. Mesenchymal and epithelial tumours of different size and different histological types arose singly or combined in one and the same experimental animal. Biological material slowed down the speed of tumour formation and decreased the diversity of tumour types, and combinations increased the amount of lymphocytic infiltrate and inhibited the process of organisation. The experimental model can be applied for testing of different cancerogenesis preventive measures.

Key words: DMBA implants, local tumorigenesis, rat model, reactive changes, foreign body.

INTRODUCTION

Experimental 7,12-Dimethylbenz(a)anthracene (DMBA) induction of cancerogenesis is well known and thoroughly investigated with the use of special experimental models. DMBA can be administered intragastrically through a probe, by local cutaneous and mucosal

application, by bronchoscopical submucosal treatment, intravenously, orally with food, by subcutaneous injections, with local intraductal mammary injections or by intramuscular injections into the neck region. Different methods for implanting cancerogenic foreign substances [1] and transplants in subcutaneous tissue [2, 3], in the spleen [4], in sublingual submucosa [5], in the bladder [6] and in the pylorus [19] have been described in experimental research.

Carcinogen pellets have been made using gelatine [8] or wax [6, 9]. Some authors have found that small doses of cancerogenic substances applied during a long term, can cause cancer much more efficiently than a single large dose given on one occasion [10, 11]. Therefore various soluble and insoluble materials including lipid matrices, particularly stearyl alcohol, cholesterol [3, 12, 13] and carbon particles [1, 3, 12] have been added to carcinogenic substrates to reduce the speed of polycyclic hydrocarbons release into the tissue.

A few authors have transplanted DMBA in biological material such as the trachea [3] or ovarian tissue [4]. Shiba *et al.* demonstrated that a higher incidence of dysplastic and neoplastic lesions was induced in the tracheal mucosa when the rate of release of DMBA had been lowered [3]. To produce malignant granulosa cell tumours, Nishida *et al.* autografted ovarian tissue containing DMBA into the spleen [4].

Tumours which arise after DMBA application originate from epithelium (carcinomas) [3, 7] and also from mesenchyma (sarcomas) [5, 4].

Usually lymphocytes are involved in antitumour immunity [21], and the intensity of immunologic reactions can affect the destiny of cell transformation [14]. Inflammatory changes have been described in DMBA models before the development of tumours [15], and an inverse relationship between the number of lymphocytes and tumour formation has been found [16]. On the other hand, in the process of foreign body tumorigenesis the inflammatory reaction is caused by the presence of a foreign body and it usually inhibits tumour formation [17]. How the inflammatory processes may intervene the combined tumorigenesis has to be elucidated.

The aim of the present study was to induce tumours, using a rat model for implanting either DMBA-containing pellets in biological material or uncovered DMBA pellets into abdominal cavity. The effects of biological tissue on the development of tumours: the time

needed for tumorigenesis, tumours dimensions, histological types and simultaneous reactive changes in implants and surrounding tissues, were studied.

MATERIALS AND METHODS

Study design

Two different experimental series were carried out, altogether on 150 white male and female rats. Tumours were induced by a cancerogen, DMBA. The reactive DMBA (C18 H10 $(CH3)_2$) was purchased from Fluka AG, Buchs SG in Switzerland. Beeswax, whale's fatty alcohol, activated carbon and a cancerogenic substance were blended, by means of warming, into a mixture. A special instrument with two halves of hollows for filling with mixture was used to shape pellets of a strictly equal diameter (2 mm) and mass (3.7 mg), each containing 0.038 mg of DMBA. An experimental model, using a foreign material encapsulated in a biological material, was applied [15].

In the first group the animals underwent bilateral adrenalectomy under ether anaesthesia. The removed adrenals were enucleated in vitro by the Evans method [18]. After the parenchyma had been removed from the organ, the DMBA pellet was inserted into the cavity to be surrounded by the adrenal capsule on all sides, lying in a biological chamber. Then both adrenals were transplanted into the abdominal cavity below the spleen, at the level of the upper apex of the left kidney (Group I).

Similar DMBA-containing pellets (without a biological chamber) were implanted just to abdominal cavity below the spleen, at the level of the upper apex of the left kidney in 72 rats without their adrenal enucleation (Group II).

The control group consisted of 50 rats. They were operated in a similar manner but the implanted pellets did not contain any DMBA. The adrenals of the rats were not enucleated either.

The animals were sacrificed after intervals of 3–4 months, 6–7 months, 9–10 months and 12 months after the implantation of the pellets. Three dimensions of the tumours were measured in centimetres and a scoring system with three levels: 1 — only microscopically diagnosed tumours; 2 — tumours of medium size (up to 13 cm); 3 —

large tumours (14–33.5 cm) was developed. Autopsy tissue samples for further histological investigation were taken from the pellet or local tumour tissue and the surrounding tissue.

Histological and immunohistochemical investigation

The tissue samples were fixed in 10% solution of neutral formaldehyde and embedded in paraffin. Histological sections were made and stained with haematoxylin and eosin in routine manner, with widely used picro fuchsin and haematoxylin after van Gieson's method, or with alcian blue.

To assess the diagnosis of sarcomas immunohistochemical staining for vimentin and desmin was performed using the streptavidin biotin (Strept ABC/HRP, DAKO Corp, Denmark) indirect staining method.

Histotopograms

The particles of pellets (foreign material), zones of adrenocortical regeneration, sclerotic connective tissue, granular tissue and lymphocytic infiltration areas around the pellet were depicted on histotopograms drawn according to the method suggested by A. Truupõld [19]. The fields of vision were scanned horizontally and vertically under a microscope with an ocular network and a preparation shifter (object-lens 8 × 0.20; ocular 7). The results of the observations (the pellet, adrenocortical regenerate, connective tissue, sclerosis, lymphocytic infiltration, granular tissue, degenerations) were drawn on a graph paper where 64 cm^2 corresponds to one field of the ocular network in the sequence they were observed under the microscope. A computerised map with areas bearing differentiating markers was drawn from these histotopograms (Figure 1). The image analysing system Image Pro 3.0 was used to analyse the maps. All fields of the maps were automatically scanned. The computer program read the map and produced the percentage for each object in the squares on the map.

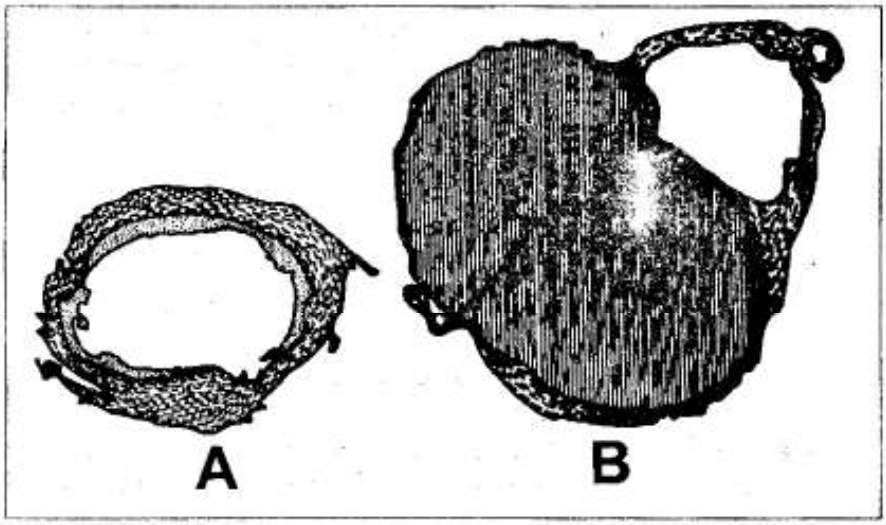


Figure 1. Histotopogram. 2 transplants 3–4 months after the beginning of the experiment (Group I). Labels: white — foreign body; striped — adrenocortical regenerate; wavy — connective tissue; dotted — lymphocytic infiltrate.

Statistics

Microsoft Excel 5.0/7.0, Sigma Statistic program, 2×2 Statistic Tables and Wilcoxon Test were used for statistical evaluation.

RESULTS

Tumorigenesis

DMBA (both in Groups I and II) induced 51 tumours in 100 experimental white rats. In Group I, where the pellets of DMBA were placed in adrenals as a biological chamber and transplanted into the abdominal cavity, there were nine rats with tumours out of 28. In Group II, where the uncovered DMBA pellet was used, there were 42 rats with tumours out of 72 (Table 1). Thus the tumour prevalence was significantly lower in Group I than in Group II ($p < 0.05$).

The first tumours were diagnosed 6–7 months after grafting the DMBA pellets. At that time the number of rats with tumours was significantly lower in Group I ($p < 0.01$). No difference between the groups in the number of tumours was found in rats sacrificed 9–10 and 12 months later (Table 1).

Table 1. Number of histological type of tumors by microscopic evaluation after DMBA pellet grafting into abdominal cavity

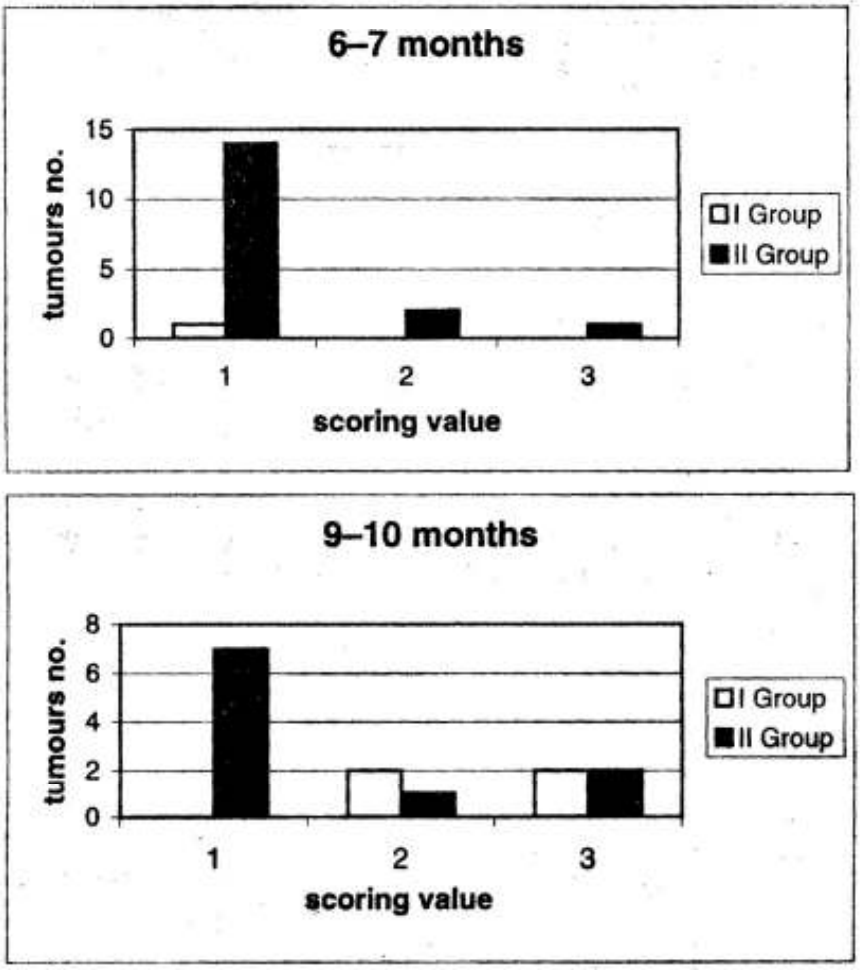
Method	n	3–4 months	n	6–7 months		n	9–10 months		n	12 months		Total n of tumours
					n			n			n	
Control Group n = 50	8	–	14	–		6	–		22	–	–	–
I Group n = 28	0/3	–	1/11[a]	*fs	1	4/9	*fs *fs + rms	3 1	4/5	*fs *fs+adc+sc	3 1	9/28[a]
II Group n = 72	0/17	–	17/19[a]	*fs *rms *fs+rms+adc *fs+adc *pleos	10 3 1 2 1	10/14	*fs *rms *pleos *fs+adc *fs+rms	5 1 2 1 1	15/22	*fs *pleos *lipos *fs+adc *fs+adc+rms	10 2 1 1 1	42/72[a]

n — number of tumours / number of rats in group
I Group — DMBA pellets in enucleated adrenals transplantated into abdominal cavity
II Group — DMBA pellets transplantated into abdominal cavity

fs — fibrosarcoma
rms — rhabdomyosarcoma
adc — adenocarcinoma
pleos — pleomorphic cell sarcoma
sc — scirrhus
lipos — liposarcoma

[a] $p < 0.01$

According to the scoring results of tumour size (Figure 2), tumorigenesis was significantly lower in Group I, than in Group II ($p < 0.01$) after 6–7 months. After 9–10 and 12 months of the experiment Groups I and II did not display any difference in tumour size.



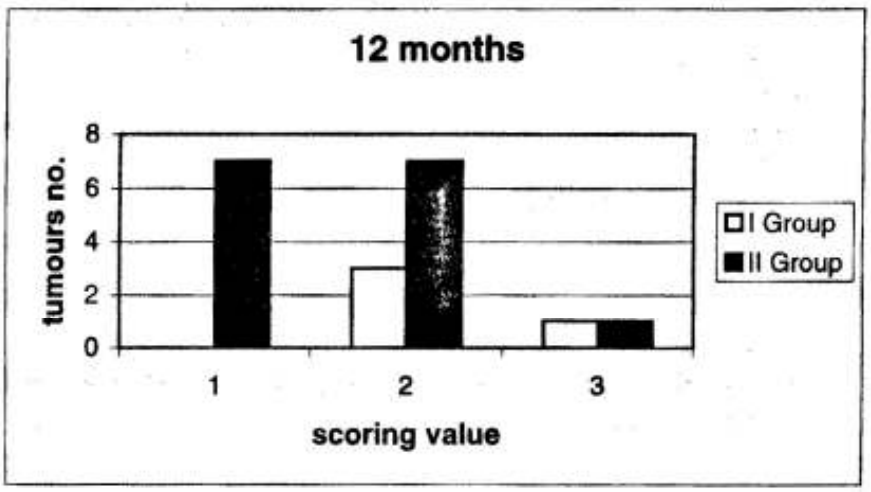


Figure 2. Scoring of tumours size in DMBA tumorigenesis after different time.

The tumours were nodular, of dense consistency, the surfaces of cut slices were whitish-greyish-pinkish with brownish and reddish nidi, at places with necrotic nidi and haemorrhage and even vacuoles filled with fluid or pus. Some larger tumours had spread into the peritoneal and retroperitoneal cavities as well as into the thoracal cavity, coalescing with spleen, intestine, left kidney and mesenterium.

Microscopically the tumours were of different histological types (Table 1). The diagnoses of fibrosarcoma and rhabdomyosarcoma were confirmed by histochemical reactions. In both groups the main type was fibrosarcoma (41 out of 51). There were eight rhabdomyosarcomas. Several combined tumours were diagnosed: fibrosarcoma with rhabdomyosarcoma, adenocarcinoma, scirrhus. In one case a liposarcoma was found. One more anaplastic tumour type was present in four cases — pleomorphic cell sarcoma. In Group II there were more different tumour types singly or in combinations than in Group I (Table 1).

Reactive changes around the implants

The pellets were either intact or were divided into segments by connective tissue. The foreign bodies presented themselves microscopically as structureless black mass, yet the pellet could not be found in

very large tumours. Usually the areas containing foreign bodies were encapsulated by the connective tissue.

Collagen fibres and their fascicles surrounded the foreign material concentrically. In some areas polyblasts, big and light epithelioid cells and fibroblasts with lymphocytes could be observed, confirming young connective tissue, e.g. granular tissue. At other places hyalinosis could be observed. Atypical cells — the beginning of tumours — could be noticed around the foreign bodies, next to or inside the connective tissue. In the control group and Group II the picture of pellet organisation was similar. Atypical cells — the beginning of tumours — could be noticed around the foreign bodies, next to or inside the connective tissue.

In Group I the connective tissue was surrounding the adrenocortical regenerate. The adrenocortical regeneration formed nodes, the differentiation of proliferated cells into a glomerular zone and a fascicular-reticular zone was observed. In control group and Group I there was less granular tissue than in Group II both in animals with and without tumours (Table 2). Organisation, i.e. proliferation of connective tissue was more markedly present in the experimental groups than in the control group (data not shown) as we noticed also in our previous paper [20].

Lymphocytic infiltration around the pellet in all periods was statistically higher in Group I as compared with the control group ($p < 0.01$).

Particularly, in Group I the lymphocytic infiltration rate in the rats was remarkably lower in the case of arising tumours (4.07, 0 and 5.08%) than without it (8.79, 8.24 and 18.16%) (Table 2).

Table 2. Reactive changes (%) by implanting DMBA containing pellet according to histotopograms

Time	Group	Median / Range			
		Granular tissue		Lymphocytic infiltration	
		Without tu	With tumour	Without tu	With tumour
3–4 months	Control	1.00/0–5.69		0*	
	I	0		7.97/2.71–13.24	
	II	12.09/0–36.74		3.45/0–20.79	
6–7 months	Control	1.64/0–13.36		1.25/0–8.41*	
	I	0	30.47/0–60.95	**8.79**/0–23,6	**4.07**/0–8.15
	II	6.75/0–13.5	3.03/0–12.85	5.05/2.34–7.77	2.79/0–10.63
9–10 months	Control	3.08/0–15.4		0.26/0–1.29*	
	I	0	0	**8.24**/1.95–13.19	**0**
	II	3.32/0–9.96	8.13/0–57.85	4.57/0–9.45	1.52/0–6.64
12 months	Control	0.42/0–5.03		0*	
	I	0	0	**18.16**/18.16	**5.08**/0–10.16
	II	16.66/0–61.78	1.75/0–6.66	4.61/0–10.55	3.24/0–19.17

* $P < 0.05$ Control Group versus Group I

DISCUSSION

We found that 0.038 g of DMBA could induce tumours which develop around the DMBA pellet in a period of 6–7 months from the start of the experiment. Our experiment induced mesenchymal and epithelial types of tumours, sometimes occurring together in one and the same experimental animal. The most frequent histological form encountered was fibrosarcoma. This is in accordance with previous studies, as tumours developing after DMBA implantation, originate either from the epithelium [3, 7] or from the mesenchyma [4, 5]. Intrasplenic DMBA grafting surrounded with biological material (ovarium tissue) has been carried out by Nishida, T. *et al.* and observed two different histological types of tumours [4].

There was no literature comparing the results of tumorigenesis and reactive changes with and without biological material. We found that grafting a DMBA pellet under the edge of spleen in a biological chamber (adrenal) or uncovered, induced the same types of local tumours. However, the number of tumours was significantly lower with biological material. This finding could be explained by the stronger immune reaction, induced by biological material.

The organisation process in its different stages from granulation tissue to hyalinosis as well as lymphocytic inflammation could be observed in the study. To understand these findings we have developed a method — histotopogram analysis.

As to reactive changes, there was more cellular infiltration around the implants with biological material, i.e. in Group I. It can be speculated that in animals with high lymphocytic infiltrate no tumours arose, particularly at the beginning of experiments. Further, in the process of tumour development the infiltration level was even less in the preparations with tumour than in the preparations without tumour. R. A. Ruggiero *et al.* have found that inflammatory reaction due to a foreign body inhibited the development of a tumour, yet the tumour in its turn reduced the inflammatory reaction induced by a foreign body [21]. Our study confirmed both findings.

In Group I there was less connective tissue and sclerosis. The connective tissue, surrounding adrenocortical regenerate, has been described in our previous studies [22]. Seemingly the regenerating biological material slows down the process of organisation of foreign material and thus tumorigenesis is also slower. In Group I tumorigenesis developed more slowly, only 5.6% of tumours formed in Group I and 94.4% in Group II in the 6–7 months of the experiment. Most of the tumours started later than 9 months after the implantation.

We compared two rat models of tumorigenesis and found that biological material with its reactions slows down DMBA-induced local tumours. When DMBA is administered systemically (a widely used model), there is a risk of mammary tumours [23]. This model enables one to study more precisely the modulating role of inflammatory processes, e.g. infections and vaccinations, on development of local tumours.

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II

Mesila I.

Mitotic activity in intact organs of rats affected by DMBA implants in the abdominal cavity.

Papers on Anthropology 1999; VIII: 127-145.

MITOTIC ACTIVITY IN INTACT ORGANS OF RATS AFFECTED BY DMBA IMPLANTS IN THE ABDOMINAL CAVITY

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ABSTRACT

DMBA-containing pellets were implanted below the spleen into the abdominal cavity of rats to give rise to a background of local epithelial and mesenchymal malignant tumours. Cell proliferation and mitotic activity was investigated in intact organs: small intestine, oesophagus, liver, the glomerular zone and the fascicular-reticular zone of the adrenal gland during a one-year experiment. DMBA causes changes in the mitotic index (MI — number of mitoses per 1000 cells), which appear regardless whether the DMBA treatment has resulted in tumour formation or not. The experiment demonstrates that the MI changes were different in different organs and mostly higher than the changes in the control group. No DMBA induced changes in cell proliferation could be observed in the oesophagus.

INTRODUCTION

Mitotic activity is one of the well-investigated indicators of cell proliferation. Cell proliferation and mitotic activity have been studied in relationship with chronobiology [9, 27, 8, 38, 44, 46], ageing [45], antibacterial treatment [44], infection [43], as observed in oesophagus and intestine, in hyperinsulinemic liver [10], in tumour by stress [52], in liver by x-ray [29]. Many authors have investigated manifestations of carcinogenic and co-carcinogenic activity in the origin and growth of tumours. Local cell proliferation increases and so does mitotic activity, which is measured and expressed by a rise in the proliferation index or the mitotic index [5, 3, 7, 40, 48, 57]. Some scientists have reported on treating tumours with inhibitory chemicals resulting in a

decrease of mitotic activity and regression of the tumour. The slowing of the tumour growth is largely due to the reduced mitotic activity [16, 20, 21, 35, 42]. The most important factor in tumour regression is hormone therapy [16, 21, 35].

Experimental Dimethylbenz(a)anthracen (DMBA) induced cancerogenesis is well known and thoroughly investigated. There are several special experimental models explaining the DMBA effects. DMBA can be administered intragastrically through a probe [1, 11, 13, 19, 32, 33, 49, 60, 62]; by local cutaneous and mucosal DMBA application [25, 58]; by intrabronchial submucosal treatment by bronchoscopy [30]; intravenously [22, 53]; orally with food [6, 26, 31]; by subcutaneous injections [14]; by local intraductal mammary injections [54, 55]; by intramuscular injections into the neck region [61]. Methods of implanting cancerogenic foreign substances [8] and transplants in subcutaneous tissue [41, 50], in the spleen [36], in sublingual submucosa [12], in the bladder [4], in the pylorus [51] have been described by authors of experimental research.

DMBA has an effect on cell proliferation and mitotic activity. DMBA induced stimulation of liver cell proliferation has been examined in small, viviparous fish who are susceptible to induction of liver tumours and measured by the mitotic index [48]. DMBA induces stimulation of the mitotic activity of melanocytes as well as cutaneus hyperpigmentation [57]. Iversen et al. in their study have shown that there is a significant difference between the influences of small and large DMBA doses. Higher DMBA doses will result in a reduction in DNA synthesis and mitotic activity in the epidermis [23]. When DMBA is applied in the solution form, mitotic activity is at first reduced because of the toxicity of DMBA [17, 59]. When a tumour develops, the rate of cell proliferation, i.e. the cell proliferation index rises [3, 59]. Apart from the well-known tumorigenic effect and cell proliferation stimulating effect, DMBA also causes inflammation [7]. DMBA treatment has been reported to simultaneously accelerate epithelial keratinization and inhibite epithelial mitotic activity [15].

All studies of mitotic activity in cancerogenesis have paid attention to the localization of the tumour. The aim of the present paper is to study the activity of cell division and to determine the level of mitotic activity in different intact organs of rats in the process of DMBA induced local cancerogenesis. Attention will also be paid to the types of tumours and to local reactive changes to the tumours and the pellet.

MATERIAL AND METHODS

The experiments were carried out on 112 white rats. Tumours were induced by a cancerogen, DMBA. The reactive DMBA ($C_{18}H_{10}(CH_3)_2$) was purchased from Fluka AG, Buchs SG in Switzerland. Beeswax, whale's fatty alcohol, activated carbon and a cancerogenic substance were blended into a mixture, and by means of a special instrument pellets of a strictly equal diameter (2 mm) and mass (3.7 mg), each containing 0.038 mg of DMBA, were shaped.

DMBA-containing pellets were implanted into the abdominal cavity of 72 rats in the Experimental Group. The pellets were placed below the spleen at the level of the upper apex of the left kidney. The Control Group consisted of 40 untreated rats. The rats in the Control Group were also operated on, but the implanted pellet did not contain DMBA.

The animals were executed after the interval of 3–4 months, 6–8 months, 9–10 months and 12 months since the implantation of the pellets. Pieces of tissue for further investigation were taken from the local tumour tissue or pellet together with the surrounding tissue, from the small intestine, from the oesophagus, from the liver and from both the glomerular and the fascicular-reticular zones of the adrenal gland.

The samples of tissue were fixed in 10% solution of neutral formalin and embedded in paraffin. Histological sections were made, which were stained with haematoxylin and eosin in routine use, with picro fuchsin and haematoxylin after van Gieson's method, with alcian blue. Immunohistochemic reactions with anti-desmin and anti-vimentin were also made. The specific intermediate filament that forms the cellular cytoskeleton was histochemically identified on DAKO tissue markers anti-vimentin and anti-desmin applied in an indirect method. Avidin-biotinyl immunohistochemical staining technique was used with DAB (3.5 diaminobenzidine) chromogen. Some of the diagnosed tumours could also be assessed macroscopically. Tumour size was measured in centimetres and all tumours were graphically shown by the largest diameter.

Particles of pellets (foreign body), connective tissue with sclerosis, granulation tissue, connective tissue with pieces of foreign body inside, areas of degeneration and lymphocytic infiltration around pellets were depicted on histotopograms drawn according to the method suggested by A. Truupõld [56]. According to this method all fields of vision were scanned horizontally and vertically by means of a microscope that was provided with an ocular network and a preparation

shifter (object-lens 8×0.020; ocular 7). The results of the observations (the contours of the pellet, connective tissue, lymphocytic infiltration, granular tissue, degeneration) were marked on a graph where 64 cm^2 corresponds to one field of the ocular network. A computerised map with areas bearing differentiating markers was composed from these histotopograms. The image analysing system Image Pro 3.0 with Materials Pro was used to analyse these maps. All fields of the maps were automatically scanned. The computer program read the map and produced the percentage of different squares for every object on the map. Microsoft Excel 5.0/7.0 and Sigma Statistic ware used to calculate statistical data..

Mitotic activity was determined in the epithelium of the small intestine, in the oesophageal epithelium, in hepatocytes and in the suprarenal glomerular and fascicular-reticular zones. Proliferation was measured by counting mitotic figures in the histological sections and expressed by the mitotic index (MI), i.e. the number of mitosis per 1000 cells in ‰ in the organ. Mitoses were counted in the fields of vision (object-lens 40×0.65; ocular 7). In the small intestine cells and mitoses were counted in 50 crypts, in the adrenal glands in 50 fields of vision, in the liver in 100 fields of vision and in the oesophagus in the cross-section. All the results were statistically analysed by means of Statgraphics and Microsoft Excel 5.0/7.0. p signifies the degree of statistical significance and r the presence of correlation.

RESULTS

Background

Tumorigenesis

0.038 mg of DMBA in the Experimental Group in these experimental conditions brought about the formation of tumours in 42 rats out of 72 in the Experimental Group. No tumours were noticed 3–4 months after the beginning of the experiment. In the period of 6–8 months 17 tumours were found in the Experimental Group. Three tumours could be detected macroscopically. In the 9–10 months of the experiment 10 tumours were found in 14 experimental animals (3 macroscopically diagnosable). After 12 months 15 tumours we found in 22 animals, and 8 were macroscopically detectable.

The size of the tumours varied — from 0.5 cm in diameter to 8×7×4 cm, noticed in the ninth month from the beginning of the experiment. Figure 1 shows all macroscopically detectable tumours presented by their largest dimensions. The tumours were nodular, of dense consistency, the surface of cut slices was whitish greyish pink with brownish and reddish nidi, in places with necrotic nidi and haemorrhage and even vacuols filled with fluid or pus. Smaller nodular tumours were with greyish pink cut surface. Larger tumours had spread into the peritoneal and the retroperitoneal cavity, the thoracic cavity, and they had coalesced with spleen, intestine, left kidney and mesenterium.

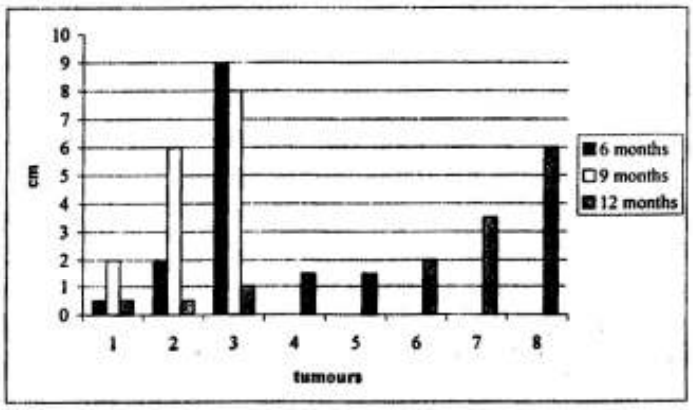

Figure 1. Macroscopic evaluation of tumorigenesis after DMBA pellet grafting into the abdominal cavity

Table 1 presents microscopic findings of tumorigenesis after DMBA grafting into the abdominal cavity. Microscopically the tumours were of different structure. In most cases the parenchyma consisted of fibroblast-like spindly cells and collagen fibres, the amount of which was different in different parts of the tumour. The cells and fibre fascicles were intersected and they were of different shape and size, the nuclei were hyperchromatic with thick chromatin lumps. Atypical mitoses were evident. Between the cells there were collagen fibres which formed fascicles of different thickness. Between the fascicles there were irregular groups of infiltrated round cells. Blood vessels and perivascular connective tissue formed stroma. On the basis of the characteristic histological structure, the diagnosis of fibrosarcoma could be given. In 32 cases out of 42 tumours fibrosarcoma was found. There were also big elongated multinuclear myosymplast-like

Table1. Microscopic types of tumours after DMBA pellet grafting into the abdominal cavity

Method	n	3–4 months	n	6–8 months	n	n	9–10 months	n	n	12 months	n	n	Total n of tumours
Control Group n=40	8	–	14	–		6	–		22	–		–	–
Experimental Group n=72	0/17	–	17/19	*fs	10	10/14	*fs	5	15/22	*fs	10		42/72
				*rms	3		*rms	1		*pleom.s	2		
				*fs+rms+adc	1		*pleom.s	1		*lipos	1		
				*fs+adc	2		*fs+adc	1		*fs+adc	1		
				*pleom.s	1		*fs+rms	1		*adc+fs+rms	1		

n — number of tumours / number of rats in the group

fs — fibrosarcoma; rms — rabdomyosarcoma; adc — adenocarcinoma; pleom.s — pleomorphic cell sarcoma; lipos — liposarcoma.

atypical formations present. Their cytoplasm was vacuolated in places and in places homogenous. Fascicles of myofibrils could be observed in myosymplasts. The nuclei were of different shape and hyperchromatic. Histological changes suggested rhabdomyosarcoma. Rhabdomyosarcoma was found in seven cases, in four cases it was single rhabdomyosarcoma and in three cases rhabdomyosarcoma occurred in combination with other types of tumour, e.g. fibrosarcoma with rhabdomyosarcoma in 1 case, in 2 other cases fibrosarcoma, rhabdomyosarcoma and adenocarcinoma with the cells located in groups, and displaying polymorphous light-coloured nuclei. In 4 cases fibrosarcoma was combined with adenocarcinoma. In one case we found fat-like tissue with irregular size of atypical cells and it was liposarcoma. One more type of tumour was observed in 5 cases of experiments. These were cases of pleomorphic cell sarcoma with cells of very different shape and size, having also hyperchromatic nuclei of different chromatin content.

Histochemical reactions with anti-vimentin and anti-desmin confirmed the diagnoses of fibrosarcoma and rhabdomyosarcoma in our study. There was a specific intermediate filament forming the cellular cytoskeleton. Antibodies reacted strongly with vimentin or desmin and labelled cells of mesenchymal or muscular origin. DAB chromagen produced an insoluble brown product that stained the nuclei of corresponding tumour cells brownly.

Reactive changes

In the preparations we also investigated what changes had taken place in the pellet and in the tissue around it. We observed that the pellets were either intact or divided into segments by connective tissue. The foreign bodies presented themselves under the microscope as structureless black mass. The areas containing foreign bodies were encapsulated by connective tissue.

Collagen fibres and their fascicles surrounded the foreign bodies concentrically. Polyblasts, big and light epithelioid cells and fibroblasts with lymphocytes could be observed in some places of the connective tissue. This was young connective tissue — granular tissue. In other places hyalinosis could be seen. Atypical cells — the beginning of tumours — could be noticed with arising tumours, around the foreign bodies, located next to or inside the connective tissue. The pellet could not be found in large tumours; it had been

encased by the tumour, which made it impossible to study the areas next to the pellet for reactive changes.

The reactive changes in the Experimental Group and the Control Group are presented in percentage in Table 2. There was more lymphocytic infiltration in the Experimental Group as compared with the Control Group (maximum 5.05%). It is of interest to mention that the rate of lymphocytic infiltration in the Experimental Group was smaller in rats with tumours (1.52%–3.24%) than the rate in rats without tumours (4.57%–5.05%), but the difference was not statistically significant.

Organisation, i.e. proliferation of connective tissue was more present in the Experimental Group than in the Control Group. The largest value was 34.91% in the Experimental Group. It was recorded 12 months after the implantation of DMBA.

Table 2. Local reactive changes (in %) to the implantation of DMBA containing pellets

	3–4 months		6–8 months			9–10 months			12 months		
	Ex	C	Ex		C	Ex		C	Ex		C
%			–	+		–	+		–	+	
1	3.45	0	5.05	2.79	1.25	4.57	1.52	0.26	4.61	3.24	0
2	12.09	1.00	6.75	3.03	1.64	3.32	8.13	3.08	16.66	1.75	0.42
3	41.95	9.06	34.04	31.30	17.43	22.88	22.72	24.52	34.91	31.49	24.19
4	37.56	72.59	54.15	39.49	52.7	69.23	43.34	52.14	43.80	54.76	45.45
5	0.07	0.27	0	0.55	0	0	0	0	0	0	0
6	1.93	17	0	21.05	26.98	0	24.46	0	0	8.75	33.37

1 lymphocytic infiltration; 2 granulation tissue; 3 connective tissue and sclerosis; 4 foreign bodies; 5 haemorrhages, necroses, degenerations; 6 connective tissue inside pieces of foreign bodies; – without tumor; + with tumor; Ex experimental group; C control group

Mitotic activity

Data expressing the level of mitotic activity in different organs both in the Experimental and the Control Groups expressed by the mitotic index (MI) are given in ‰ in Table 3.

MI in the small intestine in the third months from the beginning of the experiment was 81.2‰. In comparison with the Control Group (MI=74.1‰) the statistical difference was not significant ($p>0.05$). But in the sixth and ninth months a significant rise in the mitotic

activity in the epithelial cells could be observed, the MI values being 147.0‰ and 119.3‰ respectively ($p<0.001$). By the twelfth month the mitotic activity had dropped back to the level of the Control Group and even lower, being 56.6‰. The difference between the Control Group and the Experimental Group was statistically significant ($p<0.01$). Figure 2A presents the range of changes.

No statistically significant changes took place in the oesophageal epithelium. There was a small decrease present in the Experimental and the Control Groups, yet it was not statistically significant ($p>0.05$) (Figure 2B).

After 3 months from the beginning of the experiment, mitotic activity of hepatocytes in the Experimental Group (MI = 1.4‰) was close to that in the Control Group (MI=1.3‰) ($p>0.05$), but afterwards it began to rise, remaining on a higher level than in the Control Group (MI=3.9–4.6‰, $p<0.001$) (Figure 2C).

In the zones of the adrenal gland the changes has a different nature. In the glomerular zone, a significant rise occurred in the third month of the experiment. The MI in the Experimental Group was 0.64‰ and 0.25‰ in the Control Group ($p<0.05$). Then the mitotic activity of the Experimental Group decreased to the level of the Control Group; another rise took place in the twelfth month of the experiment (Figure 2D). In the fascicular-reticular zone a significant rise in the MI value occurred only in the twelfth month of the experiment — the MI was 0.91‰ in the Experimental Group and 0.46‰ in the Control Group ($p<0.05$) (Figure 2E).

We also studied the correlation of mitotic activity. The mitotic index of different organs shows single cases of both positive and negative correlations between the MI values of the small intestine and of the other organs: so in the third month of the experiment the correlation of MI(small intestine) and MI(liver) $r=0,609$, $p<0,01$; MI(small intestine) and MI(glomerular zone of the adrenal gland) $r=-0.588$, $p<0.05$; in the sixth month of the experiment the correlation of MI(small intestine) and MI(liver) $r=-0.532$, $p<0.05$; in the twelfth month of the experiment the correlation of MI(small intestine) and MI(fascicular-reticular zone the adrenal gland) $r=-0.529$, $p<0.05$.

The mitotic activity in rats with and without tumours in the Experimental Group was also compared. The only differences occurred in the correlation between the values of the liver and the fascicular-reticular zone of the adrenal gland in the twelfth month of the experi-

Table 3. Mitotic Index (in o/oo) in different stages of the experiment

		3 months	6 months	9 months	12 months	total
Number of rats	**control**	**8**	**8**	**6**	**18**	40
	experiment	**17**	**19**	**14**	**22**	72
	*with tumour		17	10	15	
	*without tumour		2	4	7	
Small intestine	control	74.1+–8.2	70.9+–4.7	77.1+–7.2	72.9+–4.9	
	experiment	81.2+–6.6	147.0+–3.2	119.3+–4.6	56.6+–3.2	
		p>0.05	p<0.001	p<0.001	p<0.01	
	*with tumour		146.7	122.8	54.6	
	*without tumour		149.5	112.1	60.7	
Oesophagus	control	17.1+–1.3	13.8+–1.3	13.8+–2.6	11.8+–1.6	
	experiment	14.3+–1.2	12.0+–1.2	11.9+–1.9	9.9+–1.0	
		p>0.05	p>0.05	p>0.005	p>0.05	
	*with tumour		12.4	12.6	8.4	
	*without tumour		8.5	10.3	13.1	
Liver	control	1.3+–0.3	1.2+–0.2	1.5+–0.2	1.3+–0.2	
	experiment	1.4+–0.2	3.9+–0.4	4.3+–0.2	4.6+–0.4	
		p>0.05	p<0.001	p<0.001	p<0.001	
	*with tumour		4	4.6	5.4	
	*without tumour		2.7	3.7	2.6	
					p<0.001	

		3 months	6 months	9 months	12 months	total
Glomerular zone	control	0.25+–0.06	0.20+–0.07	0.25+–0.02	0.22+–0.01	
	experiment	0.64+–0.09	0.24+–0.05	0.45+–0.16	0.45+–0.06	
		p<0.05	p>0.05	p>0.05	p<0.01	
	*with tumour		0.25	0.47	0.49	
	*without tumour		0.1	0.39	0.37	
Fascicular-reticular zone	control	0.46+–0.11	0.45+–0.05	0.47+–0.04	0.46+–0.03	
	experiment	0.64+–0.06	0.45+–0.05	0.47+–0.08	0.91+–0.19	
		p>0.05	p>0.05	p>0.05	p<0.05	
	*with tumour		0.46	0.43	1.2	
	*without tumour		0.39	0.57	0.28	
					p<0.05	

* with tumour — MI in the Experimental Group with tumour development,
* without tumour — MI in the Experimental Group without tumour development.

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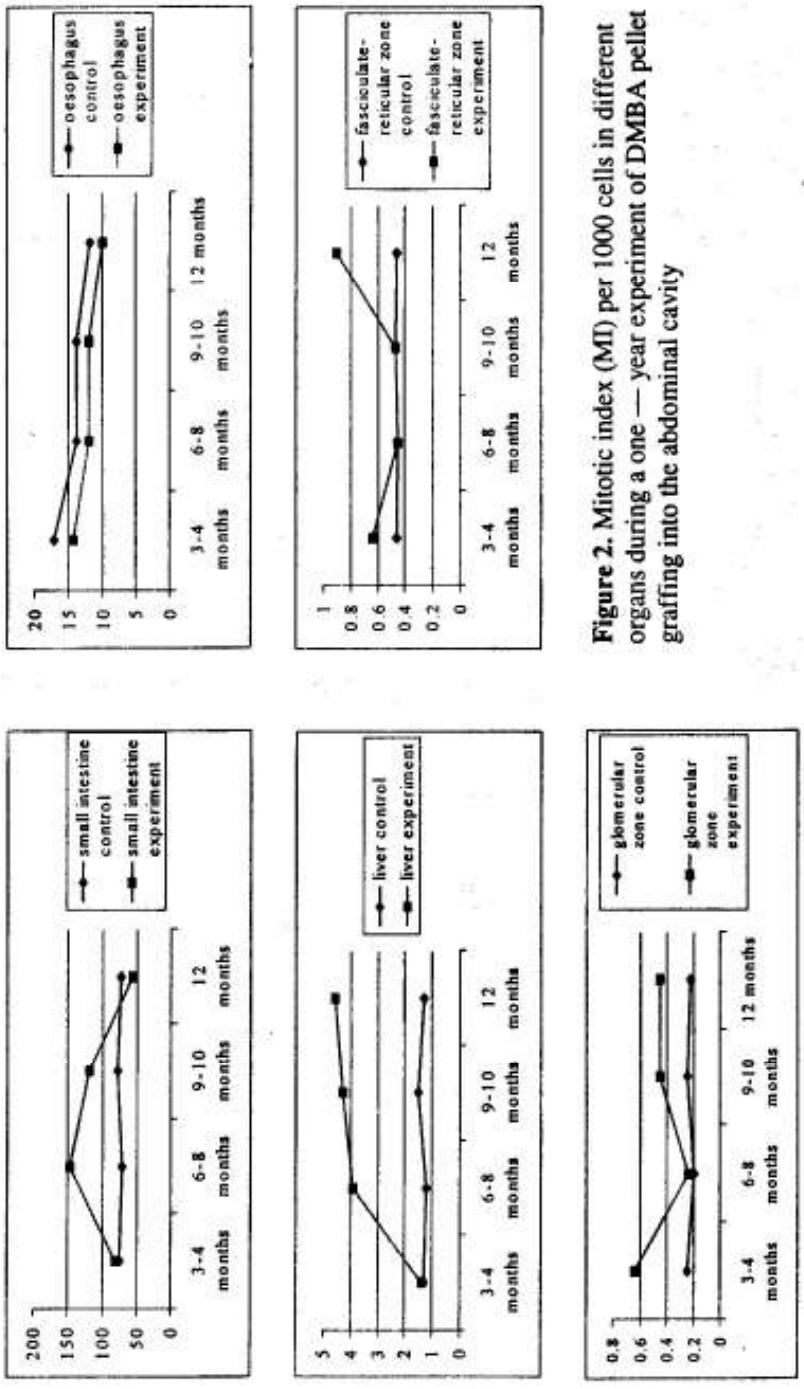

Figure 2. Mitotic index (MI) per 1000 cells in different organs during a one — year experiment of DMBA pellet grafting into the abdominal cavity

ment. The MI value for hepatocytes in rats with tumour was higher than the corresponding value in rats without tumour — 5.4‰ and 2.6‰ respectively ($p<0.001$); while comparing the values for MI in the fascicular-reticular zone the picture was about the same: in rats with tumour the MI was 1.2‰, in rats without tumour — 0.28‰ ($p<0.05$).

DISCUSSION

We found that DMBA induces changes in mitotic activity. From other studies we had learned that DMBA was able to induce local changes in mitotic activity. Some authors had found MI that increases [3, 5, 24, 30, 37, 40, 48, 57], while some other authors had found that mitotic activity decreases [15, 17]. In our paper we have mentioned that different organs react differently to DMBA presence in the organism. A persistent rise in mitotic activity was observed in the liver. Iversen et al. have reported that higher doses of DMBA will lead to an initial reduction in mitotic activity [23]. At first the decrease is due to the toxicity of DMBA and damage caused by DMBA, and later an increase in mitotic activity takes place [15, 17, 59] .Shultz et. al. found that the mitotic index was the highest on the final days of the experiment, and some doses could even be lethal to the viviparous fish, but sublethal doses would increase mitotic activity [48]. 0.038 mg of DMBA did not cause visible intoxication in rats. The high MI values in the liver may be due to the detoxication process. The data demonstrate that all changes in mitotic activity occurred locally. But our investigation is the first to suggest that the DMBA effect on cell proliferation can be wider as we could observe different changes in mitotic activity take place in intact organs far from the place of DMBA treatment.

Independent changes took place in the organs. There was no general correlation between the MI values of different organs. MI values were lower in the first months of the experiment. Stress by inhibition of mitotic activity can explain the finding, and higher mitotic indexes can be seen when the organs began to recover from the stress [52]. It is also possible that the amount of active DMBA may be small, as the DMBA release from carbon pellets is slow. No cell proliferation was observed in the oesophagus after DMBA implantation. But MI values changed in the small intestine, the liver and the adrenal glands. The

MI values in the small intestine rise for a long time, but finally cell proliferation is inhibited. Pozharisski found an increase in the mitotic index in the large intestine in the third month after the systemic application (injections) of DMBA [39]. Similar MI increases in the intestine have been found after injections of other cancerogenic agents [2]. The MI values in the liver rose and then continued high. Changes in MI values did not depend on whether tumours developed or not after DMBA treatment, the only exceptions being the liver and the fascicular-reticular zone of the adrenal gland which had higher MI values in rats with tumours. MI values did not depend on whether the tumour was macroscopic or microscopic, i.e., they did not depend on the size of the tumour.

The background in the study of MI values by DMBA tumorigenesis included different types of localised malignant mesenchymal and epithelial tumours which had developed around DMBA pellets. We found that 0.038 g of DMBA would induce the formation of tumours in a period of 6 months from the start of the experiment under our experimental conditions. The tumours developing after DMBA implantation originated from the epithelium [50, 51] and from the mesenchyma [12, 36, 61]. Our experiment induced mesenchymal and epithelial types of tumours occurring together in one and the same experimental animal. The most frequent histological form encountered in our experiment was fibrosarcoma.

Organisation process in its different stages from granulation tissue to hyalinosis as well as lymphocytic inflammation could be observed in the study. These reactive changes were very similar to those reported on in our previous studies where DMBA pellets had been implanted in enucleated adrenal glands and later transplanted into the abdominal cavity [34]. Inflammatory reaction due to a foreign body inhibited the development of a tumour, the tumour in its turn reduced the inflammatory reaction induced by the foreign body [47]. The idea that there is a relationship between inflammatory processes and the development of cancer is as old as its elusive. There exists a number of possible mechanisms, e.g. macrophages are also thought to influence cancer development in addition to their role in the generation of active oxygen radicals [18]. DMBA has the ability of inducing inflammation [7].

The results of this study suggest that DMBA promotes systemic cell proliferation regardless the magnitude and kind of reactive changes to DMBA or tumours that may develop due to DMBA influence.

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III

Mesila I.

Reactive histological changes in DMBA-containing implants grafted into enucleated adrenals of rats.

A. Rauberi mälestuskonverents, Tartu Ülikool, 1991: 37-48.

REACTIVE HISTOLOGICAL CHANGES IN DMBA-CONTAINING IMPLANTS GRAFTED INTO ENUCLEATED ADRENALS OF RATS

I.Mesila

Department of pathological anatomy

Experimental cancerogenesis, due to the effect of 1.2 diamethylbenz(a)anthracene (DMBA), has been thoroughly investigated. In a series of experiments methods have been worked out to implant into test animals cancerogenic foreign substances (10) or the abovementioned substancec together with gelatine (17), wax (7,15), wax and fats (18,22), wax and carbon particles (18,10). Some authors (14,21) have found out that small doses of cancerogenic substances given during a long term can cause cancer much more probably than a single large dose given on one occasion only. Therefore various substances must be added to cancerogenic stuff and wax in order to slow down the release of cancerogenic material.

The aim of present paper is to study the reactive histological changes in enucleated adrenals that have been implanted into the abdominal cavity together with pellets of wax and DMBA. The experimental model for pellets has been worked out at the chair of pathological anatomy of Tartu University, and has been used in the study of the structure of foreign material in enucleated adrenals employed as a biological chamber (6).

The essence of the model is the following: beeswax, whale's fatty alcohol, activated carbon and cancerogenic material are blended into a mixture and by means of a special instrument pellets of a strictly equal diameter (2mm) and mass (3,7mg), containing 0,038 mg of DMBA, are fashioned.

In the experiments 38 rats were used. The rats underwent bilateral adrenalectomy under ether narcosis.

The removed adrenals were enucleated in vitro after Evans (12). After the removal of the organ's parenchyma the pellet was inserted into the cavity in such a way that it would be surrounded by the adrenal capsula on all sides, wholly. Subsequently both adrenals were transplanted into abdominal cavity under the edge of the spleen, level with the upper apex of the left kidney.

The animals were sacrificed after 4,6,8,10 and 12 months had passed since the implantation of the pellets. The material was fixed in 10% solution of neutral formalin. From the transplants histological, 7 micrometers thick paraffin slices were formed, which were then stained with haematoxylin and eosin, van Gieson's picro fuchsin and haematoxylin and with alcain blue.

In the preparations we can see particles of pellets and zones of adrenocortical regeneration, connective tissue and lymphocytic infiltration. These areas were depicted on histotopogrammes, drawn according to a method suggested by A.Truupõld (3). According to this method all fields of vision were scanned horisontally and vertically by means of a microscope that was provided with an ocular network and preparation shifter. Precisely in the same order the results of the observations (the contours of the pellet, adrenocortical regenerate, connective tissue and infiltration nidi) were marked on graph paper where 64 cm2 corresponds to one field of the ocular network (object-lens 8 x 0,020; ocular 7).

After four months had passed since the operation, we observed that foreign material had been preserved either as an intact pellet or had been divided by the connective tissue into segments (Fig.1). Microscopically this foreign material can be seen as a structureless black mass. The areas containing foreign material are surrounded by connective tissue that surrounds them like a capsule. Collagen fibres and their fascicles surround the foreign material concentrically. In the connective tissue, further from the foreign material, we can see polyblasts, big and light epithelioid cells and spindly fibroblasts and fibrocytes. As young connective tissue cells are visible in connective tissue (polyblasts and epithelioid cells), we may conclude that a process in which new connective tissue is being formed is under way.

Connective tissue is also surrounding adrenocortical regenerate

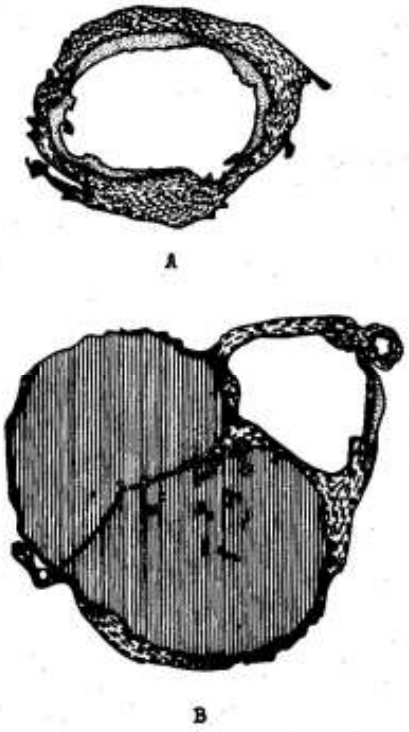

Figure 1. 4 months after the beginning of the experiment
Labels:

white - foreign material
striped - adrenocortical regenerate
wavy - connective tissue
dotted - lymphocytic infiltrate

and invades it in the form of partitions. The adrenal gland as an organ is not fully restored. The adrenocortical regeneration occurs in the form of nodes. It is not possible to distinguish the capsule from the proliferating connective tissue. We can observe the differentiation of proliferated cells into a glomerular zone and a fascicular-reticular zone which make up the better part of the regeneration. The cells of the glomerular zone are round-oval, small, dark - with a dark nucleus and contain a small amount of light cytoplasm. The ncells of the fascicular-reticular zone are large, polygonal, with a big bladder-like nucleus which contains large nuclei and small chromatin granules. Cytoplasm is mostly light in colour and thickly vacuolated. At the same time there can be found in this zone cells that contain dark cytoplasm. There occur also cells with pyknotic nuclei. The regenerate contains likewise dilated capillaries and microhaemorrhages.

Six months later the foreign material in the transplants has been preserved to a different degree (Fig.2). If we compare the histotopogrammes in Fig.2, we can see that in some cases comparatively little of the foreign material has been preserved (Fig.2,b). In some other case, thanks to the organization process, there occurs a rampant growth of connective tissue (Fig.2,e). Differently from the previous time period, the connective tissue capsula round the foreign material is laminate in structure. In the immediate vicinity of the foreign material connective tissue has hyalinised, more peripherally next to it collagen fibres and their fascicles lie concentrically. Still further from the foreign material collagen fibres and their fascicles are irregularly located, and here connective tissue has a slower rate of maturation. In comparison with the previous time period (4months), the amount of connective tissue formed is considerably large (compare Fig.1 and 2).

In structure, the adrenocortical regenerate resembles the one decribed in the previous time period. There are plenty of dilated capillaries and haemorrhages. In one case (Fig.2,d) big,light cells with vague contours can be seen in the middle of the adrenocortical tissue mass - an amorphous area of small granules. Here we have to do with dystrophic and necrobiotic changes in the adrenals under conditions of functional overstrain, a phenomenon

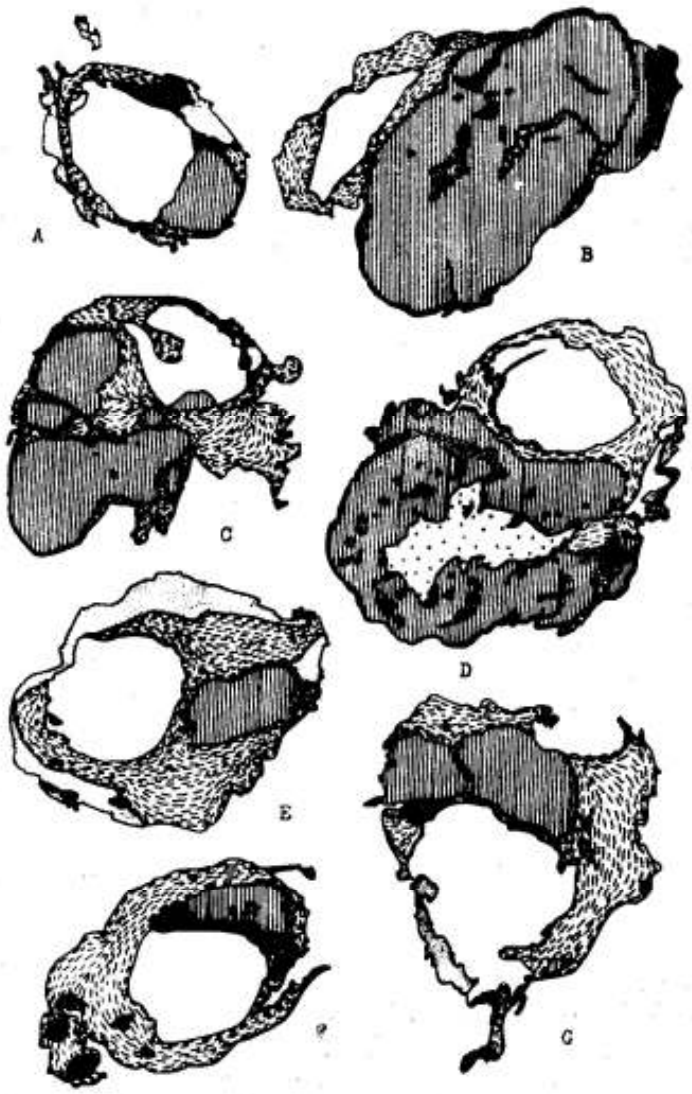

Figure 2. 6 months after the beginning of the experiment

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decribed by A. Truupôld in his writings (2,3,4).

When 8 months has passed since the start of the experiment, we observed that the changes in the transplants were extremely various (Fig.3). The foreign material has become organized to a different degree and the amount of the preserved foreign material is different in different cases (Fig.3,c and f). In some cases the foreign material pellet has remained intact, though (Fig.3,j) in parts of the transplants the material is divided into segments by connective tissue (Fig.3,d). In one case out of six we may see cells in some places in connective tissue that are atypical in nature. In comparison with normal fibrocytes these cells are big, oval, with oval chromatin-rich nuclei. There occur atypical mitoses where chromatin has been divided between two large new cells unequally. Collagen fibres and their fascicles are not regularly situated. Changes of this kind are typical of those observed in cases of rampant immature tumours of fibrous connective tissue - fibrosarcoma.

The structure of the adrenocortical regenerate is analogous to the one described in the previous time periode. There occur regenerate nodes in several masses, surrounded by a fibrous tissue as a rule. In one case the regenerate adrenocortical tissue is directly adjacent to the foreign material, in another case it is quite far from the foreign material pellet (Fig.3,a-d). In the case of one test animal the preparations were made from four pieces of different materials of which 2 are mainly composed of the regenerate and 2 contain foreign material with connective tissue.

Ten months after the start of the experiment the description of the foreign material coincides with those observed during the previous time periods. We find the same connective tissue capsule surrounding the pellet; partitions emerging from the capsule divide the foreign material into parts of different size.

But in the connective tissue around the foreign material there are clearly distinguishable rampant tumours in 4 out of 9 rats. The tumours are detectable microscopically, and in 3 cases also macroscopically.

Macroscopically the tumours are of different size and structure. In one case, in the left abdominal cavity around the spleen three

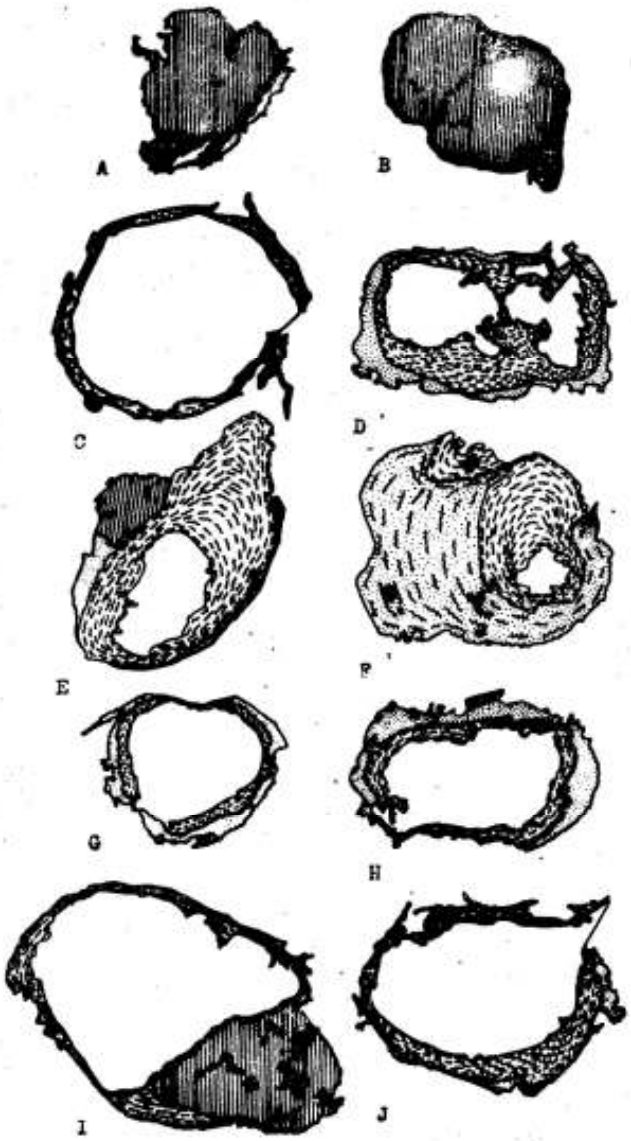

Figure 3. 8 months after the beginning of the experiment

light pink nodular formations of 0,5cm diameter can be noticed. In another case we have a greyish pink nodular structure (2x2x1cm), coalesced with the spleen. In a third case the tumour's dimensions were 8x4,5x5cm, and it had coalesced with the spleen, the stomach, the small intestine and the left kidney. On its surface can be distinguished white greyish and white pinkish nodular formations of different size. The tumour is of dense consistency, the surface of cut slices is greyish pink, alternately there occur brownish and reddish nidi. In places the necrotic nidi have become so distintegrated that there have developed vacuoles filled with fluid; the biggest bein of 3x4cm and is filled with a reddish brown turbid fluid. Microscopically it can be seen that parenchyma is made up of fibroblast-like spindly cells and collagen fibres the amount of which is different in different parts of the tumour. The cells and fibres fascicles that are intersected in different shape and size, the nuclei are hyperchromatic, with thick chromatin lumps. Atypical mitoses are evident. Between the cells there are collagen fibres which from fascicles of different thickness. Between the fascicles there are irregular groups of infiltrated round cells, plenty of them. Blood vessels and perivascular connective tissue serve as stroma. On the basis of this characteristic histological structure a diagnosis of fibrosarcoma can be given. In parallel with the described picture, in one case groups of small, round, oval or spindly cells and big elongated multinuclear myosymplast-like atypical formations are in evidence. Their cytoplasm is vacuolated in places, and in places homogeneous. In myosymplasts fascicles of myofibrils can be observed. The nuclei are of different shape, hyperchromatic. Small nuclei can be observed, there are also atypical mitoses. Histological changes resemble rhabdomyocarcoma.

The masses of adrenocortical regeneration during this time period are comparatively large and their characteristics coincide with the previous reports.

Twelve months after the beginning of the experiment the changes observed in the transplants are of so different a nature that it is more expedient to deal with all the five cases separately.

In one case we can see that the foreign material is surrounded by

normalconnective tissue.

Microscopically it is observed that in three cases there have developed similar rampant tumours of 0,5cm diameter in the region near the spleen. On microscopic examination it can be seen that in these three cases the foreign material pellet has been completely organized, i.e. it has been replaced by a hyalinised and fibrous tissue. Next to this area we can see that in two cases in connective tissue there occurin places atypical cells, whichcould be observed already in the experiments of 8 months duration. In one case there has developed a rampant tumour of infiltrate growth which is analogous to the one decribed above. In the given case we have also to do with fibrosarcoma.

Of specialinterest in the fifth case where the tumour was located on the left retroperitoneally in the form of two adjacent conglomerates (7x5 and 5x5cm). It left internal organs intact, but coalesced with the peritoneum. Macroscopically it looked like a formation having whitish, pinkish or greyish nodes of different size; its cross sections surfaces were of the same colour. Inside the tumour pus-filled vacuoles can be observed. The third rampant tumour, whitish pink with a diameter of 0,5cm is situated to the right in the abdominal cavity in the region below the liver, attached to the peritoneum. Under the microscope atypical,glandiform formations can be observed besides the above decribed fibrosarcoma. The cells are located in groups, they are polymorphous. There occur both small, dark cells between which there is plenty of connective tissue troma and large pale cells with loght-coloured nuclei. Here we can notice progressive growth of epithelial tumours. Side by side with more clearly differentiated adenocarcinoma we notice a less differentiated, anaplasic tumour - scirrhus.

The adrenocortical regenerate has developed in the same way as the one in the previous time period; it has been divided into connective tissue nodes, it has dilated capillaries and there occur microhaemorrhages as well. No traces of tumorous growth could be found.

In conclusion it can be said that 0,038g of DMBA in our experimental conditions brings about the formation of tumorous cells characteristic of fibrosarcoma after 8 months from the start of the

experiment. In case of 10-months experiments, it was possible to ascertain that in addition to microscopic tumorous changes there occured likewise macroscopic rampant tumours. After a period of 12 months, out of five cases four have an immature connective tissue tumour in different stages of development. Microscopically we have to do with fibrosarcoma which has grown of the connective tissue surrounding the foreign material. In medical literature there are reference to very different origins of tumours in different test animals after administration of DMBA in different ways. There are data about the development of sarcoma in differant animals upon the administration Of DMBA (1,8,9,13). In our experiments on one occasion we had histologically, besides fibrosarcoma also a case of rhabdomyosarcoma, and on another occasion we had a case of adenocarcinoma and a case of scirrhus. In medical literature one can find references to the development of rhabdomyosarcoma in hamsters upon intramuscular administration of cancerogenicsubstances (5), and to the development of leiomyosarcoma in mice upon implantation of cancerogenic substances subcutaneously (9). In rats adenocarcinoma has developed in mammary glands upon intravenous administration of DMBA (20), and also upon introduction DMBA into the stomach by means of a sound (11,16). After the foreign material had been transplanted together with DMBA, there is a reference to the development of carcinoma in the tracheal transplant (19).

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