Precancerous morphologic and functional aberrations in the rat mammary glands carcinogenesis

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Abstract. The precancerous changes of mammary glands in 7,12-dimethylbenz(α)anthracene (DMBA) induced carcinogenesis in Wistar rats were examined. Carcinogen was inserted into the left fifth mammary gland of the anesthetised rats. After 35 days all the animals were sacrificed and mammary glands were extirpated. Macroscopic examination of mammary glands was performed and the tissue was processed for a pathohistological analysis. H&E, VanGieson's and Toluidine-blue methods were applied, as well as ABC immunohistochemical method with anti-cytokeratin antibodies.The identified precancerous changes resembled to aberrations of fibrocystic disease in women. The fibrosclerosis, lobular hyperplasia, cystic ductal dilatation and apocrine metaplasia of ductal epithelium were found. Micropapillomatoid hyperplasia was another frequent finding, but the presence of the real papilloma was not registered. The keratocysts with the squamous epithelial metaplasia were present in three of the animals. Dysplastic changes were found in the skin, above the treated glands. The difference in expression of cytokeratins, between normal and preneoplastic epithelium, makes cytokeratin useful for verification of early precancerous lesions. The epithelial ductus and ductulus cells of the mammary glands of animals belonging to the control group showed neither CK 19 nor CK 14 expression.

Key words: Rat — Mammary gland — Carcinogenesis — Precancerous lesions — Cytokeratins

Introduction

Mammary gland is one of the most complex "target" organs. The growth, secretion, differentiation, lactogenesis and galactogenesis are under the constant influence of "interplay" of ovarian and adrenal steroids, as well as of pituitary and thyroid hormones (Macejova et al. 2005; Nilsson and Dabrosin 2006; Rajkumar et al. 2006; Applanat et al. 2008). Various morphologic units out of which a breast is made, are the base for a numerous and heterogeneous group of diseases. These diseases have high incidence and are the precursors of cancer frequently, in both the reproductive and postmenopausal period (Jmor et al. 2002; Applanat et al. 2008; Bertelsen et al. 2008).

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According to epidemiological data the breast carcinoma represents the most frequent malignant neoplasm. One of eight women bears the risk to develop this kind of tumour (Abeloff et al. 2004). The various experimental models for the examination of breast tumorogenesis (Thompson and Singh 2000) are made, with the main aim to improve the disease prevention.

Having in mind that the five year survival is up to 97% if the cancer is diagnosed at an early stage (Rosen 2001), the key goal of our work is to identify precancerous lesions and to perform an immunohistochemical examination of cytoskeletal polypeptides in them.

For the purposes of our research we used the well known and successful model of mammary gland cancer induction with 7,12-dimethylbenz(α)anthracene (DMBA) (Huggins et al. 1961; Russo and Russo 1987, 2000; Russo et al. 1990; Padmavathi et al. 2006).

Materials and Methods

Animals

The study was carried out on 27 female, virgin albino Wistar rats (weighting 120 ± 10 g), 35–37 day of age. The animals were raised in controlled laboratory conditions (in an animal room with a 12 h light/12 h dark cycle, at 22 ± 2°C). The animals had free access to the laboratory chow and tap water *ad libitum*. All procedures on animals followed Guideline for work on experimental animals approved by Ethic Committee of Faculty of Medicine in Kragujevac.

Experimental protocol

The rats were divided in two groups: the control group and the experimental group. Nine animals of control group did not undergo the treatment.

In the experimental group were 18 animals. The mixture DMBA carcinogen (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and cholesterol-buffer (serving as a vehicle) was implanted by incision in the fifth left mammary gland of the anesthetised rats. The amount of the implanted mixture for each rat was 2 mg (1 mg of DMBA and 1 mg of cholesterol-buffer). After the implantation, the incision was closed with the surgery stitch.

After 35 days all the animals were sacrificed and their left fifth mammary glands were extirpated. Macroscopic examinations and a diameter measurement of extirpated mammary glands were performed. The mammary glands were fixed in a Bouin solution for 24 h.

Micromorphologic examination

The complete material underwent routine preparations and was embedded in paraffin. The 4 μm cuttings then went through the classic H&E methods for lesion verification, histochemical VanGieson's and Gommori methods for the presentation of collagen and reticuline fibres, Toluidine-blue method for mastocyte detection and ABC immunohistochemical technique with specific antibody to Cytokeratin 14 (CK 14) (Cymbus, 1 : 200) and Cytokeratin 19 (CK 19) (ABCAM, 1 : 400).

Results

Macroscopic analysis

The animals of control group had mammary glands of the equal dimensions, 0.3–0.5 cm in diameter. Mammary glands were white-yellow colour, with a glassy look of sections and solid consistence.

The mammary glands of animals from experimental group were enlarged, 0.7–1 cm in diameter, clearly separated from the surrounding tissue and very solid consistency. They were covered with macroscopically intact skin.

Micromorphologic analysis

The microscopic examination of the mammary glands of the animals from the control group showed no pathologic changes.

The micromorphologic examination of the mammary glands from the experimental group verified a spectra of hyperplastic-dysplastic changes on the level of ductulus, ductus and acinus, frequently giving the picture of adenomatosis. A malignant process was not found.

Severe hyperplasia of smaller ductuses and ductules was followed by nuclear hyperchromasia. Hyperplastic acini are present as epitheliosis very often. In some animals, lobulus and ductulus hyperplasia is more expressed and gives the picture of microadenoma.

Larger ductuses are dilated, lengthened and curved with frequently present micropapillomatosis. Along with the papillomatosis, a cystic ductus dilatation with flattened epithelium was found. In nine animals was found cystic ductus hyperplasia with apocrine metaplasia, as well as dysplastic epitheliosis and micropapillomatosis (Fig. 1). These animals had an impressive "grape-like" hyperplasia of the myoepithelial cells in larger dilated ductuses.

Keratocysts were found in three animals. They were characterized by cystic ducts with non-structural content, small keratin bodies in lumen and the squamous epithelial metaplasia (Fig. 2).

Fibroplasia was present not only in stroma around ductuses, but also interlobulary and intralobulary (Fig. 3).

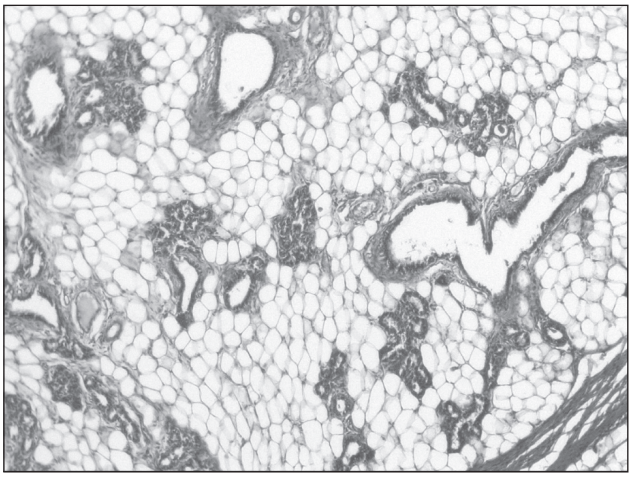

Figure 1. Dysplastic epitheliosis and micropapillomatosis (HEX150).

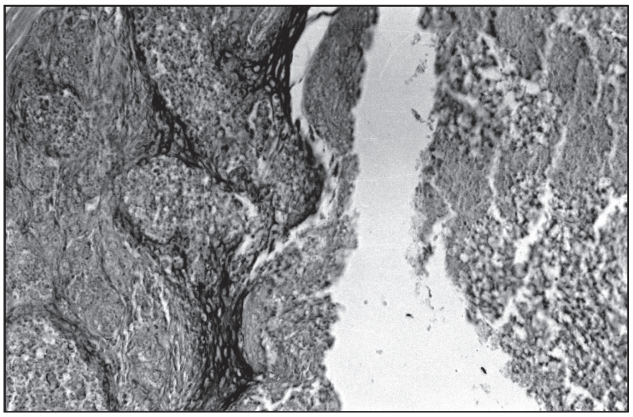

Figure 2. Cystic ductus with squamous methaplastic epithelium and necrotic content in the lumen (ABCX250).

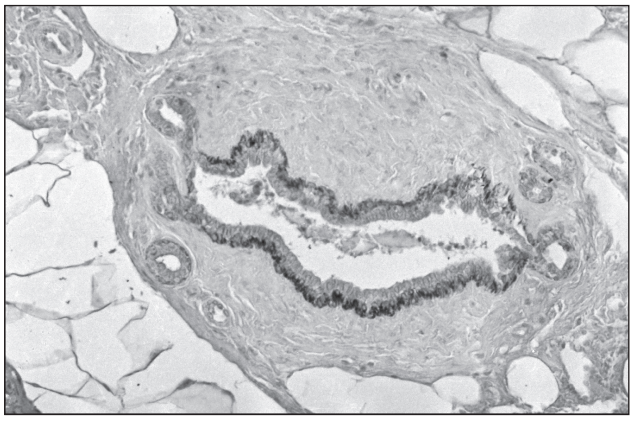

Figure 3. Strong immunoreactivity to cytokeratins of myoepithelial cells in cystic ductus (ABCX250).

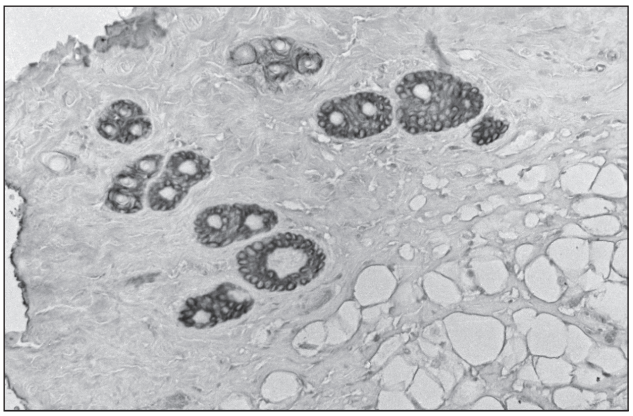

Figure 4. Strong immunoreactivity of displastic cells of ductal and ductular epithelium to cytokeratins (ABCX200).

Rich lymphoplasmocytic infiltrate had no germinative centres. Hyperplastic ductules and acini were surrounded with numerous hypergranulated polymorph mastocytes in lipomatous stroma.

Dysplastic changes accompanied by dyskeratosis, hyperkeratosis, parakeratosis and the hyperplasia of granular layer were found in stratified squamoid skin epithelium.

Immunohistochemical analysis

In the mammary glands of animals of the control group, more than 95% of myoepithelial cells of acinus showed a strong CK 14 expression (++). In epithelial ductus cells neither CK 14 nor CK 19 (–) expression was verified.

In the animals of the experimental group, in hyperplastic acini and ductules are found rare cells with heterogeneous CK 19 expression (+ to ++). In numerous dysplastic cells of ductus and ductulus a strong CK 19 (+++) expression was also present (Fig. 4). In cystic ductuses, more than 95% of myoepithelial cells showed homogenous and clearly expressed immunoreactivity to CK 14 antibodies (++ to +++) (Fig. 3). In the foci of intraductal papillomatosis the cells had predominant perinuclear content of CK 19 (+ to ++). In the foci of squamous metaplasia was present moderately strong immunoreactivity to CK 19 (++) (Fig. 2).

Discussion

In 1961, Huggins and his associates described rat mammary gland carcinoma induced by DMBA and N-methyl-N-nitrosourea chemical carcinogens. Having in mind that this method of chemical carcinogenesis of mammary glands has proven to be very efficient and, with small modifications, used by many researchers for years (Russo and Russo 1987; Korkola and Archer 1999; Leung et al. 2003), we decided to employ it for our experiment.

The examinations related to the animal age showed that the receptiveness of rat mammary gland for carcinogen is the highest in the young rats. In the older animals spontaneous mammary gland tumours are found more frequently.

Autohistoradiographic examination revealed that the terminal duct buds have the most intensive proliferation activity and that they bind the biggest amount of DMBA for the nuclear DNA. The differentiation of the terminal duct buds in 35–46 day of age rats was the most active. With the usage of DMBA, the frequency of cancer is the highest if it is implanted in this period of the animal's life (Russo and Russo 1978; Chandra et al. 1992; Haseman et al. 1998). The mentioned statements lead us towards using 35–37 day of age rats in our experiment.

Sprague-Dawley rats are the most often used for similar experiments, F344 Wistar rats and mice are used seldom, and in very rare cases rabbits and dogs (Russo and Russo 2000; Thompson and Singh 2000). In our experiment, DMBA and its vehicle are directly inserted into the fifth mammary gland of the animals. Although it is emphasized that Sprague-Dawley rats have an extraordinary receptiveness for DMBA carcinogen, we used Wistar rats and in all animals on 35th day we verified proliferative precancerous lesions.

Hyperplastic, dysplastic, cystic and metaplastic-squamous ductuses and acini changes present in animals treated with DMBA are known as "fibrocystic breast disease" in human pathology. The earliest descriptions of the fibrocystic disease included fibrosis, cyst genesis and epithelial proliferation (Bertelsen et al. 2008).

Today, the fibrocystic disease is considered as one of the precancerous breast diseases (Abeloff et al. 2004). In the literature is used a set of terminology issues connected to this disease. In England, in the past, the term ANDI (Aberration of normal development and involution) was used for fibrocystic disease (Hughes and Bundred 1989). The acceptance of such a term is based on the fact that the largest number of benign breast conditions represents the relatively small aberrations of the normal development process, cyclic hormone response and involution, which overlap throughout the whole life. The term "aberration" is used because it describes a spectra of changes which range from the smallest to the biggest ones.

The most frequent aberrations in fibrocystic disease are cysts, which can be microcysts and macrocysts. They are the consequence of the normal lobular involution joined by the active secretion of apocrine epithelium which is under hormonal stimulation. There is the opinion that multiple and recurrent cysts joined by a small cysts, have significantly increased risk for the breast carcinoma development (Tran and Lawson 2002).

Other aberrations adenosis, lobular hyperplasia, fibrosclerosis and sclerotic adenosis belong to the group of higher risk for breast carcinoma development. This is the case with sclerotic adenosis, which is sometimes hardly differentiated from carcinoma (Page et al. 2000; Salarieh and Sneige 2007). Abeloff et al. (2004) reported that the proliferation changes, first of all ductal and lobular hyperplasia, have a relative risk graded from 1.5 to 2. Atypical hyperplasia is connected with a much greater relative risk graded from 3 to 5. In women with the hereditary anamnesis of breast cancer atypical proliferation changes have a relative risk graded 11. These data suggest a recognizable process of a malignant transformation, starting from normal, turning into hyperplastic and then atypical epithelium, which is followed by a progression into ductal carcinoma *in situ* and ending in invasive breast carcinoma.

The precancerous changes found in our experimental animals are very similar to aberrations of fibrocystic disease found in women. Fibrosclerosis, lobular hyperplasia, cystic ductus dilatation, apocrine metaplasia of ductal epithelium are found almost in all animals. The micropapillomatosal hyperplasia of ductal epithelium was present but there were no real papilloma. None of the animals in our experiment developed sclerotic adenosis, which is also very rare in women (Rosen 2001).

In our material, keratocysts are verified with security after a positive immunohistochemical staining to keratin. In the identification of the comedo type of adenocarcinoma, noted in the literature (Russo and Russo 2000), one should be very careful having, in mind that keratocysts in lumen can contain necrotic detritus. The epithelium may be flattened at the same time, so that it could be very difficult with use of the classic H&E staining to identify it as a squamoid-cellular epithelial type. We have no data that this aberration is described in human breasts.

In the animal skin, above the region of the mammary gland into which the carcinogen has been inserted, dysplastic changes were noticed. In women with precancerous lesions this changes are not reported.

Recently, many researchers have dedicated their studies to investigation of functional activity of cytoskeletal polypeptides in various phases of neoplastic cell transformation. It is now well known that the cytokeratin expression is characteristic of a specific epithelium cell type, which can be used for the purpose of the identification of phenotype cell properties. Knowledge about clear difference between keratin expression of myoepithelial or base cells and glandular or luminal cells in breast, induced the formation of the new functional classification of breast carcinoma (Gusterson et al. 2005; Fulford et al. 2006; Laakso et al. 2006).

Examining the cytokeratin expression in our experiment, we found that the base cells did not show the CK 19 expression, while the luminal cells in all the preneoplastic lesions reacted to CK 19 with a specific antibody. The CK 19 expression in hyperplastic acinus and ductulus cells was heterogeneous both in terms of distribution and of reaction intensity. Heterogeneous reaction with CK 8, 18 and 19 antibodies was also described in case of the fibrocystic disease of human breasts. The same authors noted that the luminal epithelium of fibroadenoma was not reactive for CK 19 antibody and suggested that its value as a diagnostic discriminator is limited (Heatley et al. 1995).

The epithelial cells of ductus and ductulus of the mammary glands of animals of the control group did not show either CK 19 or CK 14 expression. In the literature, a subunit of luminal epithelial cells which do not react with CK 19 antibodies was identified. It is believed that negative luminal CK 19 cells of normal breast tissue have a good proliferation potential *in vitro*. This cell type was found in benign

proliferative diseases *in vivo* in a much larger number than in the normal breast tissue (Boecker and Büerger 2003). These discoveries contribute to the realization of the existence of various cellular compartments within a normal breast, which includes the progenitor compartment. In other words, it is assumed that the CK 19 negative cells can create a proliferation compartment with the possibility to contain stem cells. This supports the "early carcinogenesis" concept (Büerger et al. 2006).

We verified a homogenous CK 14 expression in myoepithelial cells of the mammary glands of all animals (both control and experimental). This mode of expression of cytokeratin is noticed in human breasts. With the help of different molecules (which along with the cytokeratin filament protein contain actin, myosin, S100 and p63) was differentiated "basal-like" breast carcinoma or tumours with myoepithelial phenotype (Clarke et al. 2005). At the same time, it is stated that the "basal-like" phenotype of breast carcinoma is joined with poor prognosis (Potemski et al. 2005).

In conclusion, we can emphasize that the preneoplastic aberrations in the rat mammary glands resembled the known changes in fibrocystic disease in women. However, the keratocysts and dysplastic changes in the squamous epithelium of skin above the preneoplastic lesions have not been described in human breast pathology. The difference between the secretory activity of normal and preneoplastic epithelium makes that cytokeratin is useful for identification of early precancerous lesions because the epithelial ductus and ductulus cells of the mammary glands in animals belonging to the control group showed neither CK 19 nor CK 14 expression.

Acknowledgement. This work was supported by Ministry of Science Republic of Serbia (project NO. 145072).

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