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EVALUATION OF THE CHICKEN EMBRYO CHORIOALLANTOIC MEMBRANE MODEL FOR LARYNGEAL TUMOR TRANSPLANTATION

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ABSTRACT

The laryngeal squamous cell carcinoma is the second common malignant tumor of the respiratory tract and together with recurrent respiratory papillomas represents the most common tumors of the larynx. Many experimental models are used to study the morphology of malignant tumors. The chicken chorioallantoic membrane (CAM) model is one of them. The CAM has all the nutrition needed for the piece of the transplanted tumor to survive. The aim of this study was to investigate whether the laryngeal papilloma and the laryngeal squamous cell carcinoma tissues transplanted on the chick CAM survive with their main histological features, and to determine the morphological changes of the CAM with different transplants. For the preparation of the CAM, fertilized hen eggs were put into an incubator for 3 days. Then the windows in the shell were opened. The fresh samples of tumors were transplanted on the CAM on the 7th day of incubation. After 3 days after transplantation the CAM with onplants were excised and fixed in the 10% formalin solution. Morphological changes in the control CAM and in the CAM with tumor onplants were observed using the digital camera on the OLYMPUS microscope. The results showed that the CAM with the laryngeal squamous cell carcinoma onplant was distinctly thicker than that of the control group and than the CAM with the papilloma onplant; the chorionic epithelium was thickened and appeared stratified of up to 5–6 layers and in some locations squamous keratinized; the mesenchymal cells were densely arranged under the tumor transplants. We observed that morphological changes in the thickness of the CAM and the chorionic epithelium were more obvious in the CAM under the

carcinoma transplants. After 72 hours of the tumor tissue transfer onto the membrane, the tumor cells retained their vitality and also their influence on the CAM tissues could be observed.

***Key words*:** *chorioallantoic membrane, chicken embryo, laryngeal squamous cell carcinoma, laryngeal papilloma.*

INTRODUCTION

Recurrent respiratory papillomas (RRP) represent the most common benign tumor of the larynx, in adults it constitutes about 10% of all the laryngeal tumors or about 87% of all the benign laryngeal tumors [19]. RRP manifest as mucosal, exophytic and benign neoplasms, usually consisting of irregular, multiple and cauliflower-like clusters, which have an intact basement membrane [11, 19]. The patients with RRP experience a different course of the disease. In some cases after the first presentation papillomatosis never recurs. Sometimes it presents with a mild course that recurs rarely. Others experience a severe disease causing outspread papillomas into the trachea and lungs with a possible lethal outcome [3, 9]. Moreover, RRP are associated with some risk (3–12%) of malignant transformation [19]. It is known that human papilloma virus type 11-positive patients show a more frequent incidence of malignisation. The majority of the malignant lesions are laryngeal squamous cell carcinomas (LSCC) [3, 11].

Laryngeal cancer is the second most common cancer of the respiratory tract with an estimated incidence rate of 5.1/100,000 in males worldwide in the year 2008 [15]. The behavior of the tumor and the survival rate in LSSC patients are different. The 5-year overall survival ranges from 0 to 100%, depending on the T- and N- category, the management approach, the tumor location and comorbidities. It is discussed what conditions, tumor characteristics or certain treatment approaches are responsible for the higher survival rates [15].

Consequently, different research models and *in vivo* assays for studying the behavior of laryngeal papilloma and laryngeal cancer tumors are performed. The classical assays for studying angiogenesis *in vivo* include the rabbit ear chamber, the mouse dorsal skin and the air sac, the chicken embryo chorioallantoic membrane (CAM), the iris and the avascular cornea of the rodent eye and the zebrafish [14]. The most

preferable these days are cottontail rabbit and nude mice models to investigate laryngeal papilloma and laryngeal cancer tumors [4, 5].

Nevertheless, the chicken embryo CAM is appropriate and a well-established model to study the behavior of different tumors [1, 2, 8], laryngeal as well. It offers the advantage of being a well vascularized and easily accessible medium, which serves as a gas and nourishment exchange surface. The main advantage of the CAM is quick, economical and a good matrix for the screening of the tumor growth [2, 14, 18]. The system of the CAM provides the cells with an approximately physiological support of nutrients, cytokines, hormones and vascularization as the natural tissue site [1, 8].

The aim of this study was to investigate whether the laryngeal papilloma and laryngeal squamous cell carcinoma tissues transplanted on the chick CAM survive with their main histological features, and to determine the morphological changes of the CAM with different transplants.

MATERIALS AND METHODS

Tissue samples. Fresh laryngeal papillomas (two cases) and two LSCC tissue samples were obtained from the operated patients in the Lithuanian University of Health Sciences Kaunas Clinic. These patients had clinical, histological and/or radiological diagnosis of laryngeal papillomatosis or LSSC. The fresh tumor tissue samples were carried to the laboratory in the isotonic saline solution. They were transplanted onto the chicken CAM in the period of 160 to 168 hours of the egg incubation within 45–60 minutes after the samples were obtained.

Chorioallantoic membrane model

Fertilized hen eggs (*Cobb-500*) were obtained from local the hatchery (Dovainoniu paukstynas, Lithuania) and kept in an incubator at 37.7ºC and 59–60% humidity, with continuous ventilation and while being rotated to and fro. On the third embryonic day (approximately 72 hours of incubation) 20 eggs' shells for each experimental line were sterilized with the 70% ethanol solution and the air chamber was punctured. After drilling the shells with a high speed drill above the yolk with embryo, the oval windows of about 1 cm^2 on the top of the shells were opened and covered with a transparent sterile tape to prevent dehydration and to

permit the observation of embryos. Then eggs were placed back into the incubator without rotation. On the 7th day of incubation the tumor tissue obtained directly from the operation theatre was sliced into approximately 1x2x2 mm pieces and each piece of tumor was transplanted onto the CAM, which was gently traumatized by laying a sterile strip onto the surface of the epithelium and then removing it immediately. After 72 hours after transplantation 2 to 4 eggs were opened. Embryos, if alive, were live-fixed in the 4% formalin solution. The CAMs with the adhering tumor were excised and fixed in the 10% formalin. The eggs incubated under the same conditions and the embryos processed according to the same protocol but without tumor onplants served as controls. After fixation a piece of the CAM with the tumor tissue was cut and embedded into paraffin, sliced 5μm thick and stained with hematoxylin and eosin.

Histological slides were evaluated histologically, and the morphometrical determination of changes in the CAM and the chorionic epithelium thickness was performed using the digital camera on the OLYMPUS microscope and the CellSense Dimentions softwear.

Statistical analysis

Data are given as means ±SD and were analyzed with the MS Excell and SPSS software. The normal distribution of parametrical variables was tested using the Student's *t* test. Results were considered significant at $p<0.05$.

RESULTS

Different types of laryngeal tumors were tested on the chicken CAM. The tumor tissue vitality was evaluated in the histological slides by observing the cells with nuclei and the appearance of the cytoplasm. After 72 hours of the tumor tissue transfer onto the membrane, the tumor cells retained their vitality and also their influence on the CAM tissues could be observed.

Tumor tissue histology

The papilloma consisted of multiple fragile clumps, with a thin and well-vascularized connective tissue core, a thick stratified squamous

epithelium and a continuous basement membrane. The underlying connective tissue had several mononuclear cells. We observed the mentioned structures in the tumor onplants after 72 hours of transplantation, but the mononuclear cells were located among the epithelial cells, close to the CAM and the tissue sample interface. Some loss of intercellular junctions in the papilloma epithelium, which may also be responsible for the tissue fragility, was observed as well. The fragility of the papilloma tissue could cause the detachment of the onplant from the CAM thus papilloma tissue pieces did not adhere to the CAM several times and were lost during the experiments.

The carcinomas consisted of the solid pieces of polymorphous atypical squamous epithelial cells with a large nucleus, prominent one or several nucleoli, abundant acidophilic cytoplasm. The transferred carcinoma tissue samples on the CAM never flowed away and firmly adhered to the membrane.

Morphological characteristics of CAM

The CAM under the onplanted carcinoma tissue was thickened due to the thickening of the chorionic epithelium and the mesenchymal layer under it. The chorionic epithelium was thickened and it appeared stratified of up to 5–6 layers and in some locations squamous keratinized (Figure 1 a, b). The thickness of the chorionic epithelium under carcinoma and papilloma onplants was 43.5±20.2 μm and 15.3±9.7 μm, in the neighboring sites – 30.9±12.7 μm and 8.9±2.6 μm, respectively; and even in distant from tumor sites the chorionic epithelium differed from the control CAM epithelium ($p<0.005$). In our investigation the control CAM epithelium thickness on day 10 was 5.14±0.79μm.

The thickness of the CAM under the tumor onplants of carcinoma and papilloma was 696.6±92.9 μm and 50.9±3.9 μm, at the neighboring sites 429.1±125.4 μm and 51.7±7.1 μm, respectively, $p<0.005$. The thickness of the CAM under the papilloma onplants did not differ significantly from the control CAM, but they were thicker in the neighboring and distant sites from the onplants ($p<0.005$, Figure 2).

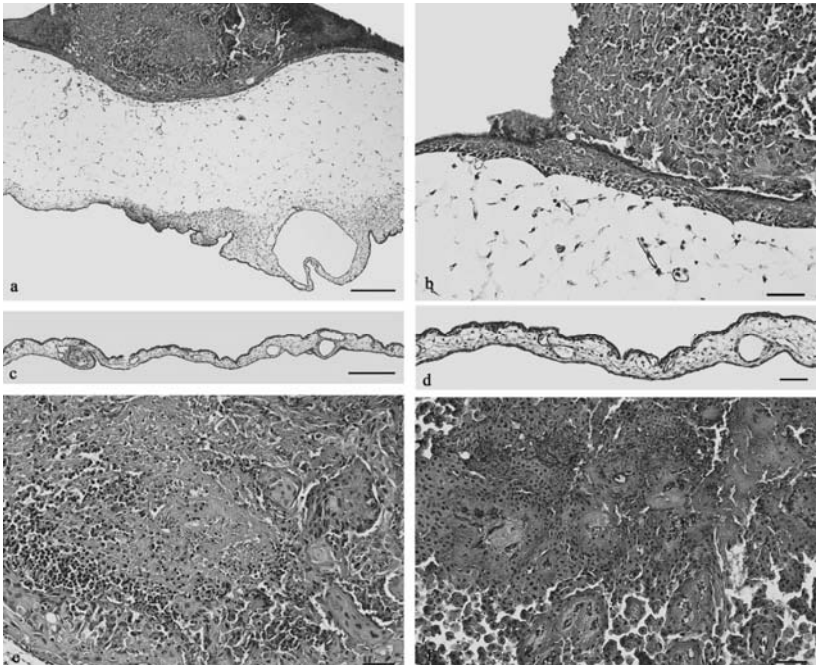

Figure 1. Densely arranged mesenchymal cells (a) and the thickened chorionic epithelium (b) of the CAM with LSCC onplant. The control CAM and its epithelium (c, d). Cells of LSCC (e) and papilloma (f) posses nuclei and vary in size and shape, i.e., retained their vitality after 72 hours of transplantation. a, c – scale bar 200 μm, original magnification 4x; b, d, e, f – scale bar 50 μm, original magnification 10x.

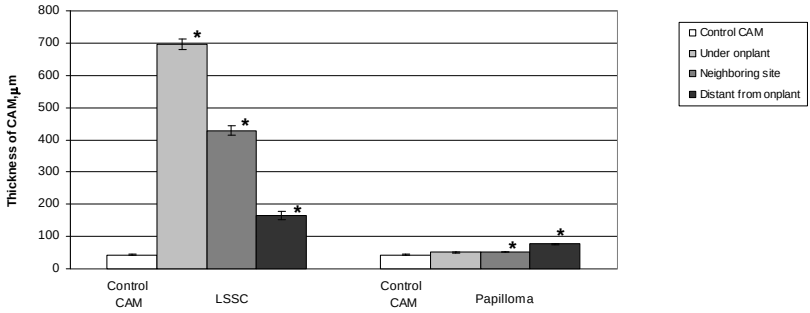

Figure 2. The thickness of the chorioallantoic membrane (CAM) with the onplants of laryngeal squamous cell carcinoma (LSCC) and papilloma (*$p<0.005$ compared to the control CAM).

The capillary network under the chorionic epithelium in the onplanted tumor region (both carcinomas and papillomas) was very rarely distributed, but in the distant sites it appeared normally dense. The large vessels appeared similarly distributed in the experimental and the control CAMs.

We noticed the difference in the arrangement of the CAM mesenchymal cells under the tumor onplants. The mesenchymal cells were densely arranged in the CAM situated under the transplanted papilloma tissue and in the neighboring sites, but in the distant locations did not differ from the control CAM mesenchyme density. Under the carcinoma onplants the mesenchyme was both loosely and densely arranged (see Figure 1a), but in distant from tumor sites it did not differ from the control.

DISCUSSION

A biological model that reflects the physiologic conditions of a solid tumor is needed for effective investigations in the cancer research. Three-dimensional in vitro models include cultured biopsies, and multilayer cultures such as cell clusters and spheroids [10].

The chick chorioallantoic membrane is a very simple extraembryonic membrane which consists of the chorionic epithelium (of ectodermal origin), the mesenchymal layer with capillaries and larger blood vessels

and the allantoic epithelium (of endodermal origin), facing the allantois cavity. By the 10th day of incubation, the CAM comprises the fully developed capillary plexus. Having a rich capillary network just under the chorionic epithelium surface, it provides the developing embryo with oxygen and calcium, and also has been employed as a model to characterize tumor growth because it can provide the transferred cells or onplanted tumor pieces necessary nutrients. The advantages of the chick embryo CAM model include the facts that: embryos can be obtained as pathogen free; until the 18th day of the incubation of tumor with host tissue is not complicated by the reactions of the host's immune system; the ability of tumor cells to traverse the chorionic epithelium and establish contact with the mesoderm beneath can be used as a convenient and easily scored end point for invasion [1].

In this series of experiments we tested a possibility to use a chicken CAM model for laryngeal tumor investigation. The results of this experiment show that both papilloma and carcinoma tumors may survive on the CAM and have an influence on the CAM itself (see Figure 1 e, f). The cells from tumor cell lines are easily transfected to the CAM, but their characteristics as tissue components are already changed, as they lack their natural interaction with one another and with other tissue components. We transplanted the pieces of tumors and observed their vitality after three days of transplantation. This shows that the CAM is able to provide enough nutrition to the tumor tissue that it remains alive. It is known that the local infiltration of the normal tissue by tumor cells and their dissemination to distant sites involve migration through the stroma of the connective tissue as well as the penetration of natural barriers, such as the basement membrane [13]. P.B. Amstrong (1982) noticed that the untraumatized chorionic epithelium is a nearly impenetrable barrier to the cells of invasive tumor lines [1]. Therefore it is very important if the CAM is damaged or not. The papilloma pieces had a tendency not to adhere to the CAM, while the pieces of carcinoma merely adhered to the CAM surface and by 72 hours already performed the influence on the CAM and induced morphological changes. A. Moscona (1959) described keratogenic metaplasia in the chorionic epithelium, which manifested itself by the alteration into the squamous stratified highly keratinized epithelium [12], but this metaplasia was due to the environmental exposure. We observed the keratogenic metaplasia in the chorionic epithelium just

beneath the onplanted carcinoma, but not the papilloma tissue pieces, and this was not observed in the control membranes or distant from the carcinoma onplant sites.

We observed the increased density of mesenchymal cells in the CAM below the tumor onplants. This may indicate that the growth stimuli, coming from the transplanted tumor tissue, can induce the proliferation and accumulation (or grouping) of mesenchymal cells. Although the thickness of the CAM was not much increased in the papilloma experiment, the increased density of mesenchymal cells was observed nearly in the whole thickness of the CAM under the papilloma onplant. We suppose that the increased density of mesenchyme cells and the thickening of the CAM and of the chorionic epithelium is the result of the CAM response to the factors coming from the onplanted tumors; and the differences in the changes of the CAMs with different tumors may depend upon the different behavior of benign and malignant tumors. Further more, each case is unique, and often the laryngeal cancer of the same differentiation and stage takes a completely different clinical progress route. That is why we could observe the different thickness of the CAM and the chorionic epithelium in carcinoma and papilloma experiments. Further investigations have to be performed to determine the factors which are derived from different tumors and their significance for the thickening of the CAM itself and for the thickening of the chorionic epithelium.

The CAM is a highly vascular extraembryonic membrane, which functions as an oxygen and calcium supplying structure; in the chicken embryo it initially appears on day 5 of incubation. From days 5 to 10, the CAM vessels progressively differentiate into capillaries, arterioles, and venules. The future capillaries' cells migrate to a position beneath the ectodermal layer of the CAM (the chorionic epithelium) and form a dense plexus of small vessels. On about day 7, the capillaries begin to migrate outward between the ectodermal cells [7]. According to the data of B.E. Dunn et al, on 10 days of incubation an electron-lucent squamous cell layer covers much of the chorionic epithelium. Intraepithelial capillaries are separated from the chorionic surface by a relatively thick (>5–μm) cytoplasmic layer [**6**].

Vascular network density in the CAM chorionic epithelium remains unchanged during the incubation. We did not notice an evident increase in the major or minor blood vessel density in the CAM in proximity of

the transplants – neither the papilloma, nor the LSCC tumor. On the contrary – this kind of carcinoma might have suppressed the appearance of new capillaries under it in the chorionic epithelium during the first 3 days of transplantation. This may also depend upon the tumor tissue expression of certain genes, which influence the new blood vessel formation and different types of cancers may express different factors; e.g. glioblastoma C6 line cells placed on the CAM in only one day attracted a dense network of blood vessels (our unpublished observation) as well as glioblastoma tissue pieces' onplants induced angiogenesis in the CAM [2].

The results of this investigation allow us to continue the research of different laryngeal tumors and to compare their invasiveness, their developmental behavior in longer experiments, the further influence on the changes of the CAM and its blood vessels. Perhaps it is not worth to prove once again that the intact epithelium is an impenetrable barrier for tumor invasion [1]. As not all papillomas gain a developmental character of a malignant tumor, it would be interesting to investigate the behavior of papilloma tumors and their invasiveness on the CAM, as well as to determine their relation to papilloma virus types, which may be responsible for recurrent laryngeal papillomatosis.

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