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A CHICKEN EMBRYO MODEL TO STUDY THE GROWTH OF HUMAN UVEAL MELANOMA

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In vitro cultured human uveal and skin melanoma cells were injected into the chicken embryonal eye at a stage when the immune system was not yet mature. The melanoma cells were accepted as part of the organism by the host. Even single melanoma cells could be traced by morphological methods as well as by immunohistochemical markers, such as S100, HMB-45, NKI/C3 and HNK-1. We found tumors in 20 and 40 percent of the embryos injected with uveal melanoma and skin melanoma, respectively. The embryos did not exhibit abnormal development of the eye as a result of the microinjection and had a high survival rate (90 and 60%, respectively) during embryogenesis. With this model for uveal melanoma the growth and possibly the metastatic behavior of human uveal melanoma cells can be studied.

The life prognosis of patients with primary uveal melanoma has not improved despite recent introduced irradiation techniques [1,2,3]. The estimated 15-year survival rate after detection of the tumor is 46 percent [4]. The mean disease-free interval for cutaneous melanomes is 14.3 years versus 22.3 years for ocular melanomas. Patients with ocular primary tumors have the highest incidence of liver metastases and the shortest subsequent survival [5,6,7].

Until now there are two types of animal models developed to study uveal melanoma. Albert and coworkers (1980) [8] were able to culture human uveal melanoma tissue experimentally in nude mice by transplanting fresh (human) tumor tissue into the

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anterior eye chamber. In the second animal model cultured primary uveal melanoma cells [9,10] were injected in the anterior and posterior chamber of rabbit eyes [8,10,11]. The xenografted rabbits, however, needed continuous immunosuppressive therapy.

The purpose of the present study was to develop an animal model to examine the growth of human melanoma cells in the eye without using immunosuppressives. To do so *in vitro* cultured human uveal and skin melanoma cells were injected into the eyes of chicken embryos before maturation of their immune system. We describe a simple model for studying the growth and possible metastatic behavior of uveal melanoma.

MATERIAL & METHODS

Embryos

Fertilized eggs of *Gallus gallus domesticus* were obtained from a local supplier and incubated in a forced draught incubator at 37°C and 60 % humidity. Embryos were staged according to the number of incubational or embryonal days (E). From E2.5 (embryonal day 2.5) onwards, the eggs were not tilted any more.

Microinjection

At E3.5, the embryonal eye is localized at the surface of the chorioallantoic membrane and easily recognized because the retinal pigment epithelium has just developed. The injection of melanoma cells into the eye with a microinjection system is therefore simple. After E3.5, the head of the chick moves farther away from the surface and dips towards the bottom of the egg. Microinjection was performed with a glass pipette with an internal diameter of 50 μm. Each microinjection resulted in the grafting of about 10^2 - 10^3 single cells of either skin or uveal melanoma into the developing eye (1 - 10 μl injection).

Culture of skin melanoma

A human skin melanoma cell line derived from a lymph node metastasis (kindly provided by the NKI, Amsterdam) was cultured in RPMI medium supplemented with 10 % heat-inactivated foetal calf serum, penicillin and streptomycin. This cell line was a fast growing monolayer culture, which underwent passage every week at 1:4 dilution and was grown continuously for more than two years. Confluent cell layers were trypsinized, washed with phosphate-buffered-saline (PBS), centrifuged, and the resulting pellet was suspended in PBS (5 X 10^6 cells/ml) for microinjection.

Culture of uveal melanoma

A 46-year-old patient who had undergone enucleation for uveal melanoma 28 years earlier suffered from metastatic lesions in the liver, kidneys and subcutaneous tissue.
After obtaining informed consent, a subcutaneous metastatic nodule was excised. At histological examination the metastatic tumors in liver, subcutaneous tissue and kidneys were consistent with metastatic amelanotic and melanotic melanoma of the mixed cell type (spindle and epithelioid cells). The tumor of the subcutaneous tissue was minced with a pair of fine scissors, irrigated continuously with Dulbecco's modified Eagle's medium (DMEM) and subsequently strained through cheese cloth. The resulting suspension consisted mostly of single cells and to a lesser extent of small clumps of tumor cells. The tissue was characterized by a diversity of small and large multinucleated cells. Most nuclei contained multiple prominent nucleoli and numerous mitotic figures. The cells were washed twice with DMEM medium supplemented with 10 % heat-inactivated foetal calf serum, penicillin and streptomycin and seeded in a culture flask at 5 % CO_2. A feeder layer was not used. The cultures, which consisted of adherent and non-adherent cells,

were maintained in DMEM medium supplemented with 10% foetal calf serum. Confluent cultures underwent passage every 2 to 3 weeks at 1:4 dilution. The adherent cells were trypsinized, washed with PBS, and centrifuged. The pellet was suspended in PBS (5 X 10^6 cells/ml) and directly used for microinjection.

Antisera

S-100 [12,13], NKI/C3 [14], HMB-45 [15] and HNK-1 [16] were used for the immunohistochemical studies of both the cell cultures and the histological sections of embryonal chicken eye. The S-100 monoclonal antibody recognizes an acidic intracellular Ca^{++} binding protein and is a non-specific marker for melanoma cells [17]. Monoclonal antibodies NKI/C3 and HMB-45 are markers for precursors of melanosomes and melanoma cells. Monoclonal antibody HNK-1 recognizes a family of cell adhesion molecules (CD57), migrating neural crest cells and a series of neural crest derivatives.

Immunohistochemistry

The chicken embryo heads were decapitated at E10 and E19 and fixed in 10 percent formalin/PBS for at least two days. The eyes were enucleated, dehydrated and embedded in paraffin. The sections, 5-7 μm thick, were prepared by staining with hematoxylin/ eosin or by incubation with the above mentioned primary antibodies in a moist incubation chamber at room temperature for 30 minutes. Negative and positive controls for the primary antibodies were included. For immunoperoxidase staining, rabbit-anti-mouse peroxidase conjugated immunoglobulins were used as a second-step antibody. Endogenous peroxidases were inhibited by incubation for 25 minutes in a methanol/hydrogen peroxide (99:1 v/v) solution. Peroxidase was visualized with 0.1 % 3,3'diaminobenzidine HCL (Serva) and 0.01 % hydrogen peroxide. Sections were counterstained with hematoxylin for one minute. PBS with 0.1 % Tween-20 was used for all rinses.

Cytocentrifuge preparations of cell cultures were analyzed like that used for histological sections.

RESULTS

Culture of skin melanoma cells

The amelanotic melanoma cells grew as a uniform monolayer culture with several mitotic figures and possessed numerous dendritic processes. The skin melanoma cells bound markers specific for melanoma-antigens for example HMB-45, NKI/C3, and S-100 (Table 1).

Table 1. Expression of cell markers on *in vitro* cultured and xenografted human melanoma cells

	S-100	HMB-45	NKI/C3	HNK-1
Metastatic uveal melanoma	n.d.	++ (100%)	+++(100%)	++(10%)
Metastatic uveal melanoma cultured *in vitro*	++(20%)	++ (70%)	++ (70%)	+++(50%)
Uveal melanoma xenografted into chicken embryonal eye	+ (60%)	+++(100%)	+ (70%)	n.d.
Skin melanoma cultured *in vitro*	+ (90%)	+ (100%)	+ (90%)	+ (100%)
Skin melanoma xenografted into chicken embryonal eye	++(100%)	- (0%)	- (0%)	+ (100%)

n.d. = not determined; Intensity of staining: -= no staining; + = slight staining; ++=intense staining; +++= very intense (saturated) staining; percentage in parentheses is the number of positive cells observed.

Culture of metastatic uveal melanoma cells

One week after initial plating of the metastatic melanoma cells about 40% of the cells became adherent and nonadherent cells were removed weekly. After 3 weeks some adherent cells became rounded and had lost the ability to adhere. Some of the non-adherent cells (i.e. 25% approx.) were vital, according to trypan blue exclusion analysis, and hence multiplied in suspension, becoming adherent again after being seeded into new flasks. The culture was kept as a suspension culture and was grown continuously for one year. To date there have not been any signs of differentiation, pigmentation or a decrease in the doubling time.

Microinjection of human skin melanoma cells

Twenty embryos were injected with skin melanoma cells; twelve embryos survived E10 and eight of these embryos developed an intraocular melanoma. The eyes were removed for histological evaluation on days E10 or E19. The chicken eye was macroscopically normal. The injection site had undergone regeneration and could not be identified histologically. Histochemical studies revealed foci of melanoma cells, which appeared vital and mitotic figures were numerous. In all eyes, the melanoma cells grew in the choroid (Fig. 1) and along the tunica vasculosa lentis. There was no growth in the vitreous body. At stage E10, large numbers of infiltrating tumor cells were already present. The melanoma cells stained strongly positive for the monoclonal antibodies S-100 and HNK-1 and stained negative for the monoclonal antibodies HMB-45 and NKI/C3 (Table 1). Even single melanoma cells were easily detectable on days E10 and E19.

Microinjection of human metastatic uveal melanoma cells

Eighteen out of twenty injected embryos survived (E19) from which four embryos (E19) exhibited intra-ocular tumor growth (Fig. 2). On histological examination the chicken eye had developed normally and the injection site could not be recognized. Metastatic uveal melanoma cells were seen adherent to the ciliary body, along the hyaloid artery and along the tunica vasculosa lentis. The tumor nodules derived from uveal melanoma were smaller than those originating from the injected cutaneous melanoma cells.

Immunohistochemistry

We determined the expression of four markers (S-100, HMB-45, NKI/C3, and HNK-1) on human skin and uveal melanoma tissue sections, on *in vitro* cultured human cells and on the xenografted melanoma cells (Table 1). Some examples are illustrated in Fig. 3. For both skin and uveal melanoma we found a difference in marker expression which depended on the growth system used. Skin melanoma cells in particular lost all marker expression for HMB-45 and NKI/C3 when xenografted into the chicken.

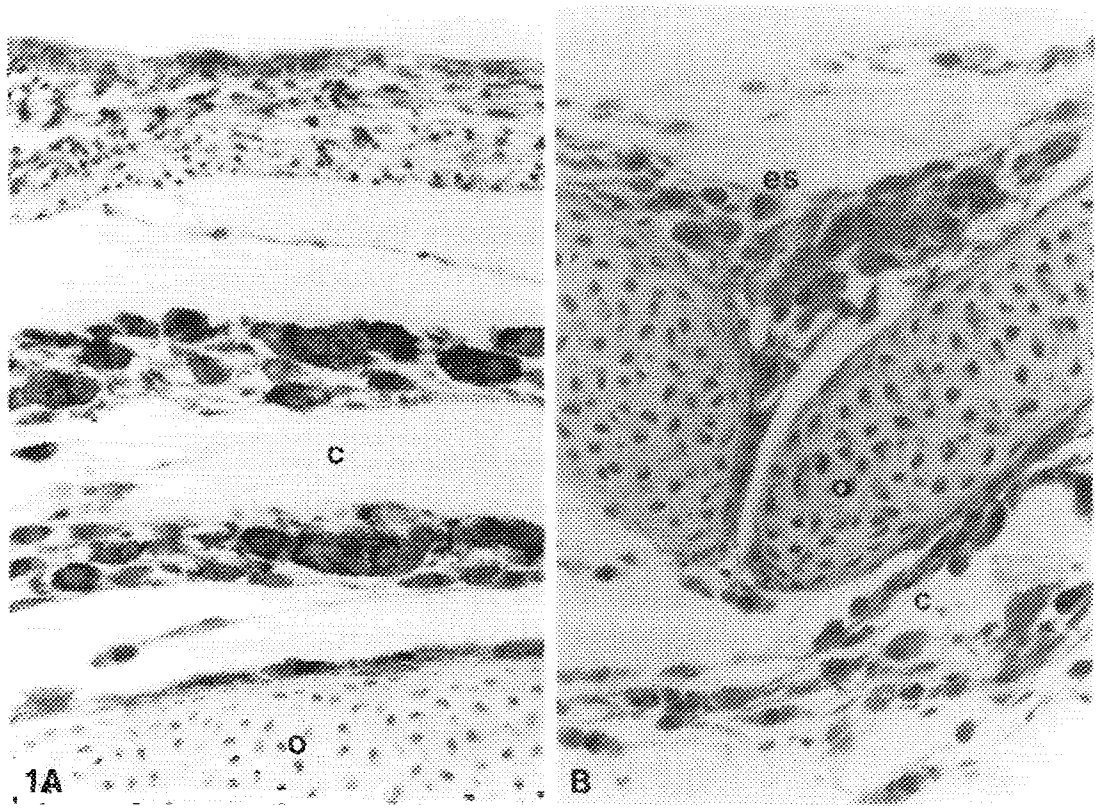
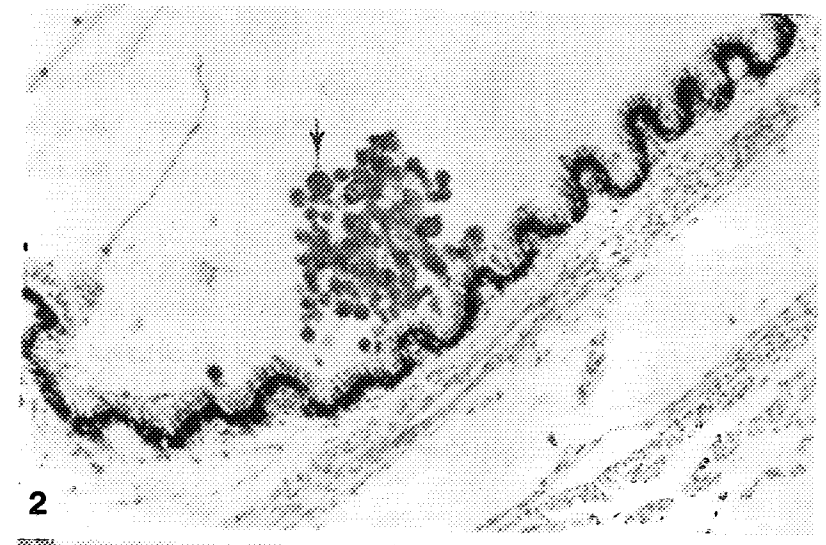


Fig. 1. Growth of cultured human skin melanoma cells injected into the eye on E3.5 and analyzed on E10. The tumor cells bind intensely the S100 antibody (A) and E19 (B). Infiltration of melanoma cells is observed in the choroid (c), between the cartilage ossicles (o), and extrascerally (es). (Magnification 320x, 420x, respectively).

Fig. 2. Growth of human uveal melanoma cells along the ciliary body in an E19 eye. The tumor cells stain intensely with the HMB-45 marker (arrow). (Magnification 520x).

Fig. 3. A) Human uveal melanoma metastasis in subcutaneous tissue stained with HMB-45; uveal melanoma (u) infiltrating lymphocytes (i) B) Cytocentrifuge preparations of metastatic human uveal melanoma cells stained with HNK-1. About 50% of cells are positive, a diversity of cell types can be seen. Several cells are multinucleated (arrows). (Magnification 100x, 320x, respectively).

In contrast to skin melanoma, xenografted human uveal melanoma retained the marker expression for HMB-45 and NKI/C3. The S-100 marker revealed no large differences in marker expression for either type of melanoma irrespective of growth conditions. We conclude from the results summarized in Table 1 that xenografted human uveal melanoma cells can be traced on histological sections of the chicken eye with the four markers and that marked change in morphology of the xenografted uveal tumor cells does not occur.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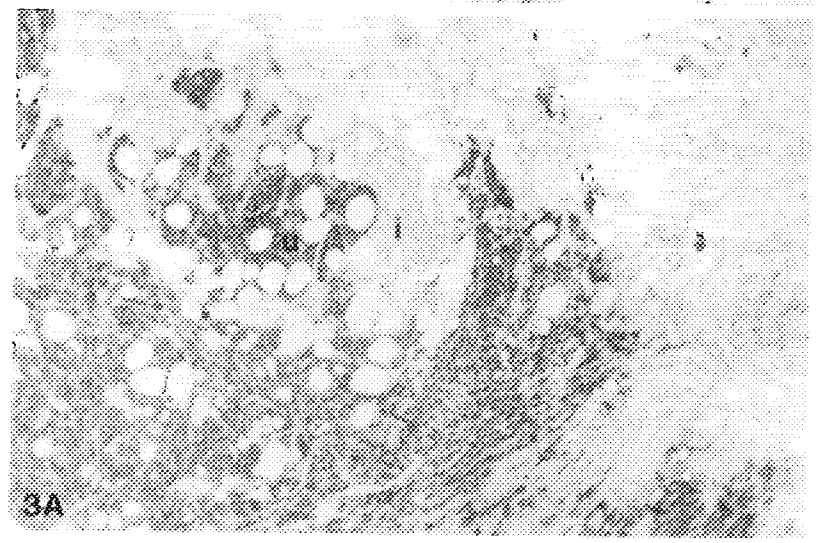
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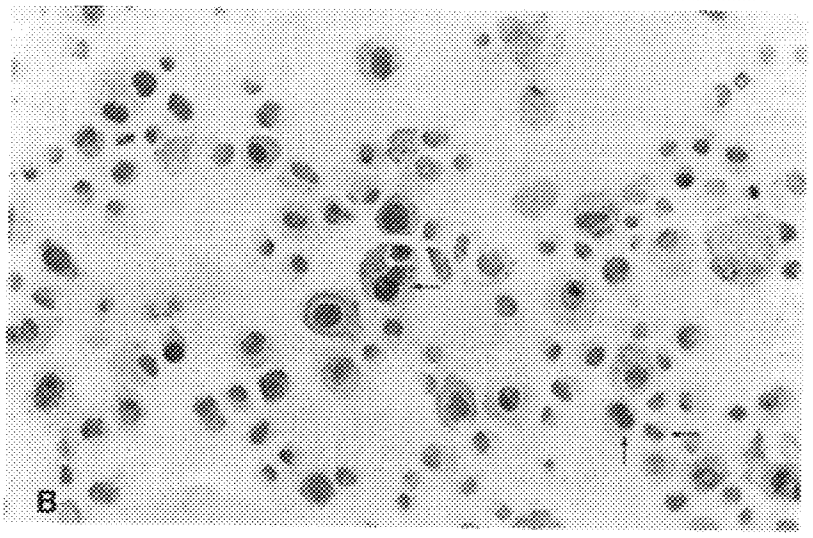
B

DISCUSSION

To our knowledge no animal model is described in which tumor cells are injected in chicken embryos before the development of their immune system. We found that the uveal and skin melanoma cells injected in the eye are tolerated by the host immune system.

The growth of the xenografted melanoma cells was rather typical, the xenografted melanoma cells preferably grow onto extracellular matrixes in different eye tissues.
There was a difference in expression of the markers between the observed tumor foci and the injected cells, possibly clonal selection had occurred *in vivo* resulting in a different marker make up. Another possibility is that the altered marker expression is environment-dependent.

This new animal model is useful, large series can be studied, and therapeutical agents can be tested. At present the possibility of metastatic behavior of in vitro cultured primary and metastatic uveal melanoma cells and genetically manipulated uveal melanoma cells in the hatched chicken is under study.

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