The chick chorioallantoic membrane: a model of short-term study of Dupuytren’s disease

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Abstract

Dupuytren’s disease is a progressive fibroproliferative disorder that impairs hand function by altering the normal structures of the palmar fascial bands. Nodules composed almost entirely of myofibroblasts and cords are pathognomonic of Dupuytren’s disease. The myofibroblasts express alpha-smooth muscle actin which is especially involved in development of the disease. We aimed to evaluate whether the xenograft of Dupuytren’s fibromatosis taken from operating room and transplanted on chick chorioallantoic membrane (CAM) survives with its histological and immunohistological features. Fresh samples obtained from eight patients with Dupuytren’s disease were minced and immediately inoculated onto 24 CAMs of 8-day-old chick embryos. The implanted CAMs were examined daily by stereomicroscopy and finally the xenografts were examined and characterized in histological sections using a panel of antibodies. The xenografts were incorporated into the CAMs 6–7 days after transplantation, continued to grow and stimulated angiogenesis in the chick embryo CAMs. The CAMs vessels entered the xenografts and anastomosed with the newly formed xenografts vessels (CD34+ and CD105+) those containing nucleated chick erythrocytes. Myofibroblasts (α-SMA+) and macrophages (CD68+) were readily recognized in the xenograft thickness. We concluded that the xenografts of Dupuytren’s fibromatosis transplanted onto chick embryo CAMs continued to develop and preserved the histological and immunohistological features.

Keywords: Dupuytren’s disease, myofibroblast, alpha-smooth muscle actin, chick chorioallantoic membrane.

Introduction

Dupuytren’s disease is classified as a soft tissue fibromatosis, a progressive fibroproliferative disease of an unknown origin that includes keloid scars, penile fibromatosis, plantar fibromatosis and fibromatosis of the proximal interphalangeal joints [1–3]. The disease affects the fascia in the subcutaneous tissues of the palm, causing a permanent flexion contracture of the fingers. The lesions can be multiple, bilateral, and may coexist in the palm and in the sole (Ledderhose’s disease) [4]. Grossly, there are ill-defined, whitish and firm nodules or cords, which microscopically are characterized by focal fibroblast proliferations that exhibit an infiltrative pattern of growth, a varied amount of dense collagenous tissue in-between, and no abnormal mitotic activity. Thus, fibroblast proliferation (Dupuytren’s nodules) and connective tissue assemblies (cords) are pathognomonic of Dupuytren’s disease [4, 5]. Regarding the burden of collagen fibers, the density of myofibroblasts and blood vessels, there have been defined three phases of evolution: proliferative, involutive and residual, with an increasing collagen deposition and decreasing cellular component. The altered fibroblast proliferation has been linked to the abnormal expression of various growth factors, which have been shown to be important in the pathobiology of Dupuytren’s disease [6, 7]. These factors may induce the differentiation of fibroblasts into myofibroblasts, which are the offending cell in Dupuytren’s disease [8]. Fibroblast to myofibroblast differentiation is characterized by alpha-smooth muscle actin (α-SMA) expression, which is the actin isoform of vascular smooth muscle cells [9, 10]. Flow cytometric analysis of the cells from Dupuytren’s nodules showed that the majority of cells were myofibroblasts and M1 phenotype activated macrophages [11].

The chick embryo CAM have been used as a model system for studying development, biomaterial’s properties, angiogenesis, photodynamic therapy, human tumoral cell metastasis, microsurgical interventions [13–17]. The chick embryo CAM is an extraembryonic membrane formed by fusion of the chorion and the allantois between day 4 and 5 of incubation, which mediates gas exchanges with the extraembryonic environment. It comprised of three layers: chorionic epithelium composed of epithelial cells derived from the chorionic ectoderm; mesoderm composed of highly vascularized mesenchymal tissue; allantoic epithelium, a derivative of the allantoic endoderm [18, 19].

Owing to its relatively immunotolerant trait, the chick embryo CAM shows the ability to efficiently support the growth of inoculated xenografts including human tissue explants for extended periods of time [17, 20, 21].

The aim of this study was to investigate whether the human xenografts of tissue fibromatosis harvested from the patients with Dupuytren’s disease and transplanted on chick embryo CAM survives with its histological and immunohistological features.

Materials and Methods

Tissue samples

Fresh samples of palmar aponeurosis placed in isotonic saline solution were obtained from patients with Dupuytren’s
disease operated in the Clinic of Plastic Surgery, Emergency County Hospital, Craiova, Romania. Patients were selected for the study after providing individual informed written consent according to the guidelines of the Ethics and Scientific Deontology Committee of the University of Medicine and Pharmacy of Craiova. Samples were minced with a microtome blade to obtain 1–2 mm³ pieces for immediately CAM inoculation. One fresh fragment from each patient was fixed in 10% buffered neutral formalin and processed for histopathological assessment.

**Chick chorioallantoic membrane model**

Fertilized White Leghorn chicken eggs were incubated at 37.5°C and 70% relative humidity. After 72 hours of incubation, the top of the eggs were cleaned with a 70% ethanol solution. Using an 18-gauge needle, 3–5 mL of albumen was extracted from each egg to detach the CAM from the shell, this allowing a small window to be cut at the top of the egg. The window was then sealed with adhesive tape and the eggs were placed back in the incubator until day 8, when the chorioallantoic membrane becomes capable of sustaining human tissue xenografts.

On day 8, one small piece of palmar aponeurosis tissue was transplanted on top of the CAM under sterile conditions and eggs were returned to the incubator until day 15 (three chick CAMs per patient sample).

CAMs were examined and registered daily by a Zeiss stereomicroscope equipped with a DCM 510 camera system. At day 15–16, the xenografts were harvested together with the surrounding CAM and fixed with 4% paraformaldehyde for 24 hours.

**Immunohistochemistry**

Fixed tissue specimens and CAMs were routinely processed for inclusion in paraffin blocks and sectioning. Sections were sliced utilizing a rotary microtome to achieve 4 μm-thick sections for both histochemistry and immunohistochemistry.

For immunohistochemistry, the slides were incubated with the primary antibodies overnight and the next day the signal was amplified utilizing either the EnVision or the Tiramide CSA II amplification systems (Dako, Medicalkit, Craiova, Romania). Briefly, after antigen retrieval, sections were cooled to room temperature and incubated for 30 minutes in a 1% hydrogen peroxide solution. The sections were next washed in phosphate buffered saline (PBS), followed by a final blocking step of 30 minutes in 1% skim milk. The primary antibodies were added in dilutions recommended by the producers, and then the slides were incubated overnight at 4°C (Table 1). Next day, slides were washed and the signal amplified utilizing one of the two systems described above according to producer’s recommendations. After washing, the signal was detected with 3,3'-diaminobenzidine (DAB) (Dako). All intermediate washing steps were done in 0.1 M PBS, pH 7.2, and all antibodies were diluted in PBS with 1% bovine serum albumin (BSA) [Sigma-Aldrich, Medicalkit, Craiova, Romania]. Finally, the slides were coverslipped after a Hematoxylin staining. Routinely Hematoxylin-Eosin stained slides were also included in the study.

### Results

Fresh samples of palmar aponeurosis obtained from eight patients with Dupuytren’s disease were transplanted onto 24 CAMs of 8-day-old chick embryos. Three chicken embryos (12.5%) died during incubation period. Six samples of human fibroproliferative tissue (25%) fail to develop.

**In vivo observation**

Stereomicroscopic examination showed that four or five days after transplantation, the fragments of human fibroproliferative tissue begin to be covered by the CAM. On day 8 after grafting, the xenografts were almost completely enveloped by the CAM. The human xenograft continued to develop as the CAM vessels invaded its periphery (Figure 1B). A marked angiogenesis with new vessels arranged in a wheel-spoke pattern was visible around the grafted tissues from the 6th day of incubation (Figure 1C and D).

**Histological and immunohistochemical analysis**

Mature chick CAM was composed of three anatomically distinct layers: the chorionic epithelium with a rich network of subchorionic blood capillaries; the mesoderm with a few large blood vessels; and allantoic epithelium (Figure 2A). The chorionic epithelium and allantoic epithelium were positive for CD3 and CD20cy [Mouse, IgG1k, Dako] (Figure 2A). The chorionic epithelium and allantoic epithelium were positive for CD3 and CD20cy (Figure 2, F and G) and CAMs vessels were positive for α-SMA (Figure 2E). CAM structures were negative for CD34, CD105 and CD68 (Figure 2, B–D).

Under and especially around the xenograft area, the CAM was notably thickened (Figure 3, A and B), with numerous new mesodermal blood vessels that invaded the periphery of the xenograft.

In the xenograft thickness, the pathological features of Dupuytren’s fibromatosis were readily recognized. Mostly they consisted in intermediate-late lesions that were characterized by low cellularity and rich collagenic bands with pseudonodular disposition. Myofibroblasts were detected as positive for anti-α-SMA, but increasing stromal collagen component was predominant. Immunohistochemistry showed that the myofibroblast were distributed throughout the vascularized areas of the xenograft (Figure 4, A and B). Macrophage-like cells were

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<th>Epitope</th>
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identified in the lesion as reactive for anti-CD68 antibody. Immunohistochemistry showed that the macrophages were distributed throughout the low vascularized areas of the Dupuytren’s nodules (Figure 4, C and D).

Both human (anti-CD34 and anti-CD105 positive) and CAM vessels were detected in the xenograft tissue containing nucleated chick erythrocytes, suggesting a functional communication between the chick and the human transplant vascular systems (Figure 4, E–H).

**Discussion**

In this study, we have shown that the CAM model can be used as a simple and inexpensive method for short-term investigations of the Dupuytren’s fibroproliferative tissue development. The natural lack of immune function of the developing chick embryo allows heterogenic transplantation because xenograft rejection will not occur [17].

The angiogenesis in the surrounding CAM is an essential process for further development of the xenografts that continued to grow only after the CAM vessels entered its thickness. Lack of CAM angiogenesis was observed around the xenografts that fail to develop.

Immunohistochemical staining revealed human (CD34+) neovessels (CD105+) reperfused by anastomosis with the invading vessels of the CAM. Therefore, the development of the fibroproliferative tissue is related to the angiogenesis in the xenograft thickness.

The macrophages pattern of distribution observed in the xenograft was throughout the nodules and it was similarly with that already reported in the immunohistochemical study of the Dupuytren’s disease patient [11].

Different patterns of myofibroblasts and macrophages localization were observed in the xenograft thickness: myofibroblasts were present predominant in areas with both CD34+, CD34- vessels (anastomosing areas of the CAM vessels that enter the xenograft and human neovessels) and macrophages were present throughout the Dupuytren’s nodule around a few CD34 positive vessels (xenograft neovessels). This suggests a connection between the distribution of myofibroblasts and angiogenesis on the xenograft thickness.

![Figure 1](image)

**Figure 1** – Stereomicroscopic aspects of Dupuytren’s disease xenograft: (A) In vivo aspect of the xenograft after implantation; (B) In vivo aspect seven days after grafting (15-day-old chick embryo CAM); (C) Formalin-fixed xenograft with surrounding CAM sample, upper side; (D) Same sample, lower side.
Figure 2 – Histological (A) and immunohistochemical (B–G) aspects of the 15-day-old chick embryo CAM (scale bar, 50 μm). Ce: Chorionic epithelium; Ae: Allantoic epithelium; M: Mesenchymal layer; Mv: Mesenchymal vessel.

Figure 3 – α-SMA staining shows a thickened CAM at the junction with the xenograft (A), and around the xenograft (B), that contains numerous blood vessels. (C) Normal CAM. Scale bar, 50 μm.
Figure 4 – Immunohistochemical aspects of the CAM and xenograft tissue (scale bar, 50 μm): (A and B) α-SMA+ cells especially localized between Dupuytren’s nodule, into the vascularized areas of the xenograft; (C and D) Few CD68+ cells throughout low vascularized areas of the Dupuytren’s nodule; (E and F) CD34+ human blood forming anastomoses with the CAM vessels (CD34-). These human blood vessels were CD105+ neovessels (G and H).
This work shows that the CAM can be used as a model for in vivo study of Dupuytren’s fibromatosis. Also, the data revealed that human fibroproliferative tissue stimulated angiogenesis in the chick embryo CAM.

References


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