Establishing Efficient Xenograft Models with Intrinsic Vascularisation for Growing Primary Human Low-grade Sarcomas

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Abstract. *Background: There are no xenograft models of low-grade soft tissue sarcoma. Transplant survival remains an obstacle in sarcoma xenograft models and is attributed to post-transplantation hypoxia. Models with an intrinsic tissue - engineered vascular supply may overcome this obstacle. The aim of this study was to establish a novel xenograft model of primary human low grade soft tissue sarcoma. Materials and Methods: Primary low-grade liposarcoma fragments were transplanted into a silicon chamber, placed around the superficial epigastric vessels in athymic nude mice. Xenograft samples were assessed histologically (light/electron microscopy and immunohistochemistry for S100). Results: All xenotransplants of low grade primary soft tissue liposarcoma (n=4) engrafted, led to the development of solid tumours in mice. Histological and immunohistochemical staining confirmed the xenografts as being well-differentiated liposarcomas identical to the original tumor tissue. Conclusion: Successful transplantation of human low-grade liposarcoma tissue in mice was established for the first time using a model with an intrinsic vascular supply.*

Soft tissue sarcomas are a heterogenous group of tumours derived from primitive mesenchymal cells. The most common subtype is liposarcoma, which represents 24% of extremity and 45% of retroperitoneal soft tissue sarcomas. Liposarcomas are predominantly a disease of adulthood, with peak incidence around the fifth to sixth decade of life and with a slight predominance toward males (1, 2). In adult patients, the well-differentiated subtype is the most commonly encountered (3-5). Histological subtype (as well as tumour location) is one of the most important predictors of outcome. Dedifferentiated, pleomorphic and round-cell liposarcomas are high-grade, aggressive tumors with metastatic potential, whereas well-differentiated liposarcomas are low-grade tumours that follow a more indolent clinical course (3-5). While these categories represent various points on a spectrum of disease, each of these entities displays its own unique character. The most common subtype (50% of liposarcomas) is the well-differentiated; which are known to recur locally and have a risk of dedifferentiation (2-5).

Complete surgical resection remains the mainstay of local therapy, but adjuvant radiation therapy is effective at controlling microscopic residual disease after surgical resection (6-11). Local tumor recurrence remains a clinical problem in the treatment of low grade soft tissue sarcomas (4, 5, 7, 12). Treatment for sarcomas has lagged behind for more common types of epithelial cancer and survival from liposarcoma has remained unchanged for several decades (13, 14).

The use of xenografts of human tumors in nude mice is an accepted model for *in vivo* biological and preclinical studies (13-19). Xenotransplantation has become a widely used tool both in order to demonstrate the tumorigenicity of cells and to test the efficacy of therapeutic interventions *in vivo* (20). The stability and comparability regarding phenotype, differentiation and characteristics of malignancy are

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important prerequisites of any tumor model. The subcutaneous implantation of tumour fragments into immunocompromised nude mice is a widely accepted model for the study of various tumor types (13-19) but transplantations frequently fail, in particular for low-grade sarcomas and xenografts may be established from the more aggressive tumor subtypes and thus, may not represent the whole spectrum of soft tissue sarcomas (13, 14). Hypoxic conditions after transplantation may in part be responsible for the cytological instability of xenotransplants and we hypothesised that primary soft tissue sarcoma models with an intrinsic tissue engineered vascular supply would help overcome this obstacle. The aim of this study was to establish a xenograft model of primary human low-grade soft tissue sarcoma with an intrinsic tissue engineered vascular supply in nude mice.

Materials and Methods

Animals. The animals used were NMR nude mice (Harlan Winkelmann GmbH, Borchen, Germany). The mice (n=4) were sexually mature males, 6 weeks old and weighed about 20-25 g. They were housed in ventilated racks, under pathogen-free conditions under a 12 h light-dark photoperiod and with controlled humidity and temperature (20±2˚C). Boxes, bedding, food and water were sterilised. Animal care and manipulation was in agreement with institutional guidelines and the Guide for the Care and Use of Laboratory Animals (21). All animal experiments were carried out under the guidelines and with the permission of the Ethics Committee of the Ruhr University Bochum. All activities were performed under sterile conditions and general anaesthesia. The tumor specimens were obtained with written informed consent of patients and with the permission of the Ethics Committee of the Ruhr University of Bochum.

Isolation of sarcoma specimens for xenotransplantation. Soft tissue sarcoma tissue was directly transferred from the operating theatres of our hospital to the laboratory. The tumor samples were taken under sterile conditions from representative areas of the original tumor mass. The samples were then divided into two adjacent parts, one for further histology (conventional light/electron microscopy and immunohistochemistry) and the other for the *in vivo* experiment. The tumor samples were sliced into 1-2 mm fragments prior to transplantation.

Vascularised groin chamber and in vivo sarcoma xenograft model. Tissue engineering chambers consisting of 5 mm of length of silicone laboratory tubing (3.35 mm internal diameter, 4.4 ml volume; Dow-Corning Corp., Midland, MI, USA) were inserted into the left groin of each mouse as described previously (22, 23). The NMR nude mouse was placed in a supine opposition, the groin of the mouse were exposed and a transverse incision parallel to the inguinal ligament was performed. The inguinal fat pad and the superficial epigastric artery and vein were dissected for 1 cm of length from their origin at the femoral vessels.

The cylindrical tissue engineering chamber was then placed around the vessels and anchored to the underlying muscle with a 10-0 nylon microsuture. The proximal aperture and longitudinal seam of the chamber were sealed with bone wax (Ethicon, Somerville, NJ, USA). Care was taken to avoid occlusion of the vascular pedicle. The chamber was filled with tumor fragments suspended in Matrigel® (BD Bioscience, Palo Alto, CA, USA). The matrix solidifies at 37˚C and thus ensures that the cells remain *in situ*. Next, the distal aperture of the chamber was also sealed, creating a closed chamber again taking care not to compromise the vascular patency. The construct was then carefully returned to the dissected space within the groin, and the wound was closed (Figure 1). All animals survived the anaesthetic protocol and recovered well. Animal behaviour returned to normal and remained unsuspicious. No wound breakdown was noted.

Chamber harvest and specimen assessment. After an *in vivo* incubation of 3 weeks in which the animals were allowed to move freely and were fed a standard mouse chew *ad libitum*, the wounds were reopened and the tissue engineering chamber was exposed. The vascular patency in the chamber was recorded. The proximal and distal parts of the vascular pedicle were cut outside the chamber and the chamber was removed from the implantation site. The surrounding fibrous capsular was resected from the outside and the chamber was reopened. The newly formed tissue was then carefully removed. Macroscopic images were taken and the tissue was sliced into two sections, one for histological assessment the other for electron microscopy. Tumor pathology was analysed by histological and immunohistochemical staining.

Histological, immunohistochemical assessment. Tumor samples were stored in 10% formaldehyde. At least five individual sections of each primary tumor were assessed. The primary tumor and the xenotransplant counterpart were sectioned into 5 μm-thick sections.

Samples of the tumors were fixed in 10% buffered formalin (PathoMed, Viersen, Germany). At least five individual sections of the primary tumor were assessed. For histological evaluation, the sections of the primary tumor and the xenotransplant counterpart were deparaffinised, rehydrated and stained with haematoxylin and eosin following standard procedures to assess cell and nuclear morphology. Immunohistochemical staining was carried out using a mouse anti-S100 (DCS clone 15E2E2; Millipore; Billerica, MA, USA) antibody at a 1:500 dilution according to the manufactures guidelines.

Electron microscopy. Tumor samples from the primary tumor as well as the xenotransplant were fixed in 2% glutaraldehyde and embedded in Epon 812. Uranyl acetate and lead citrate were added to the ultrathin sections for contrast. Specimens were analysed with special focus on differentiation criteria (1). Microscopic features of the tumors such as cellularity, growth pattern, cytomorphology, vascularity, invasiveness, degree of differentiation, and necrosis were recorded.

Results

Diagnosis of the primary human low-grade liposarcoma was confirmed by independent reference histology. Tumor diagnosis and classification were determined according to WHO guidelines (24).

Histological and electron microscopic morphology of the primary tumors. The well-differentiated liposarcomas were histological characterized by a typical growth pattern similar

to that of benign lipomas (Figure 2a). Mature adipocytes showed a significant variation in cell size and focal nuclear atypia next to scattered stromal cells with hyperchromatic nuclei and *fibrous septa*. Lipoblasts were also frequently found. Immunohistochemistry showed an expected positive reaction with S100. Ultrastructurally, the specimens were characterized by lipomatous cells containing numerous lipid globules varying in number and size. Dark and light lipid vacuoles were present on electron microscopy. Rough endoplasmic reticulum was elongated with dilated cisternae. Heterogeneity of tumor cells and nuclei was found. Nucleoli were often small and the number of nucleoli was not markely increased. A single necrotic area was noted (Figure 2b-d).

Histological and electron microscopical morphology of the xenotransplants. Four specimens from well-differentiated liposarcomas were analyzed. All specimens displayed a vital tumor with slight inflammation and focal fibrosis. One case showed strong inflammation with tumor necrosis up to 20%. The other cases had only a slight inflammation, with circumscribed necrosis (Figure 3a). In the electron microscopic analysis of the chamber specimens half of the specimens, showed signs of cell necrosis. Heterogeneity of tumor cells and nuclei was obvious. Nuclear irregularities in size and shape were observed and tumor cells contained numerous lipid globules, varying in both number and size compared to the original tumor. In all specimens, it was frequently noted that there was accumulation of numerous lipid vacuoles in the tumor cells. Dark and light lipid vacuoles were present on electron microscopy. Nuclear irregularities, with an increase in large nuclei, were seen. Rough endoplastic reticulum was elongated with dilated cisternae. Calcifications, interpretated as degenerative signs, were found sporadically (Figure 3b-d).

Discussion

This preliminary study demonstrates successful transplantation of primary human low-grade liposarcoma tissue in nude mice for the first time ever *in vivo* using a model with an intrinsic tissue-engineered vascular supply. Xenotransplantation of tumor cells into immune-deficient mice is well recognised and is a useful experimental model in cancer research (13, 14, 16-18, 25), but the high failure rate after transplantation remains an unsolved problem, particularly for low-grade tumors (13, 14). Hypoxic conditions after transplantation may in part be responsible for the cytological instability of such xenotransplants and our intrinsic tissue-engineered vascular supply overcomes this obstacle. In a large comparative study of human sarcoma xenografts evaluating intraperitoneal and subcutaneous transplantation sites, Hajdu *et al*. found an overall take rate of 62%, but only 51% of all tumor specimens grew subcutaneously, with 52% appearing less differentiated, 13% better differentiated and only 35% resembling the primary tumor when compared to the human tumor resection specimen (26). Intraperitoneal transplantation resulted in an even higher discordance of differentiation. It is well established that in the initial phase after transplantation cells are exposed to a hypoxic environment and rely on diffusion alone for nutrition (27-29). Hence a diffusion barrier of more than 200 μm will hamper cell survival (30). It can be hypothesised that this initial vulnerable phase can be accounted for the low graft-take after xenotransplantation and may also promote the selection of more resistant and hence more dedifferentiated tumour cells. The latter is well reflected in the observation that on the one hand the subcutaneous xenograft model is more successful with high-grade tumors and on the other hand that dedifferentiation of the tumor when compared to the human primary is frequently reported (13, 14, 31). To overcome this obstacle in the present study a vascularised chamber for cell transplantation was used. The vascularised chamber was initially developed for cell transplantation in a tissue engineering setting at the Bernard O`Brien Institute of microsurgery (22). It has been proven to successfully promote cell survival of thymus (28), cardiac muscle (27), myoblasts and various other tissues (23, 32, 33). Centered around a vascular pedicle, the chamber allows for early and rapid neoangiogenesis supporting the nourishment of the transplanted cells, while reducing the duration of hypoxia (22).

In the present study all of the low-grade liposarcoma xenotransplants in the groin chamber resulted in successful survival of the transplanted tumors. As expected from the tissue engineering experiments, increased angiogenesis was also observed after xenotransplantation of tumor tissue. All groin chambers displayed a dense functional newly formed vascular network, which contrasted with the little or no vascular observed in growth in the subcutaneous pocket specimens. Tumor cells migrated within the matrix of the groin chamber and seemed to be more evenly distributed throughout the chamber. The tissue engineering chamber produced viable and good tumor growth and seems to be a favourable model to analyze early tumor implantation and tumor-stromal interactions. It also offers the chance to better assess tumor angiogensis early after transplantation. In conclusion, consistent and successful transplantation of primary human low-grade liposarcoma tissue in nude mice was established for the first time ever *in vivo* using a model with an intrinsic tissue-engineered vascular supply.

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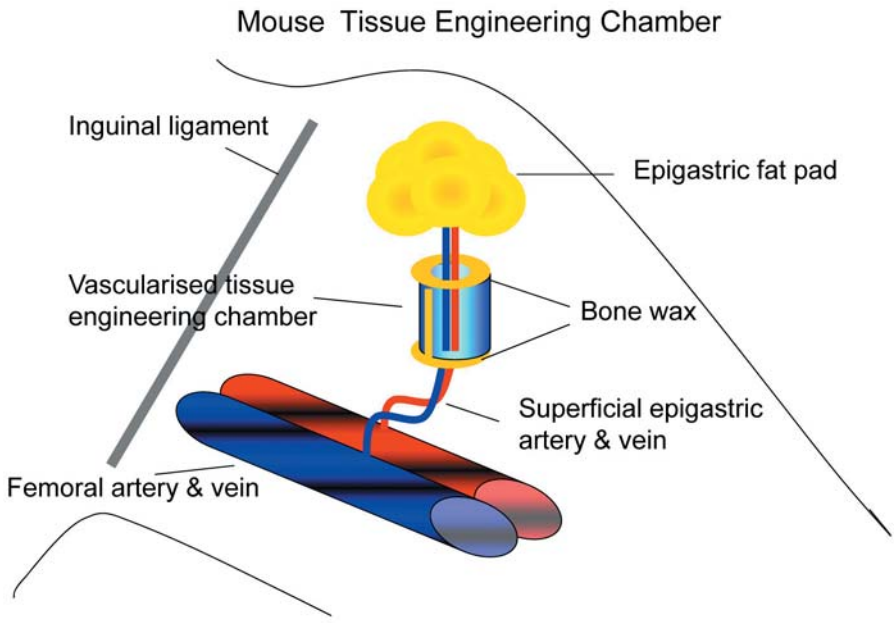

Figure 1. *Mouse vascularised tissue engineering groin chamber model for growing primary human liposarcoma.*

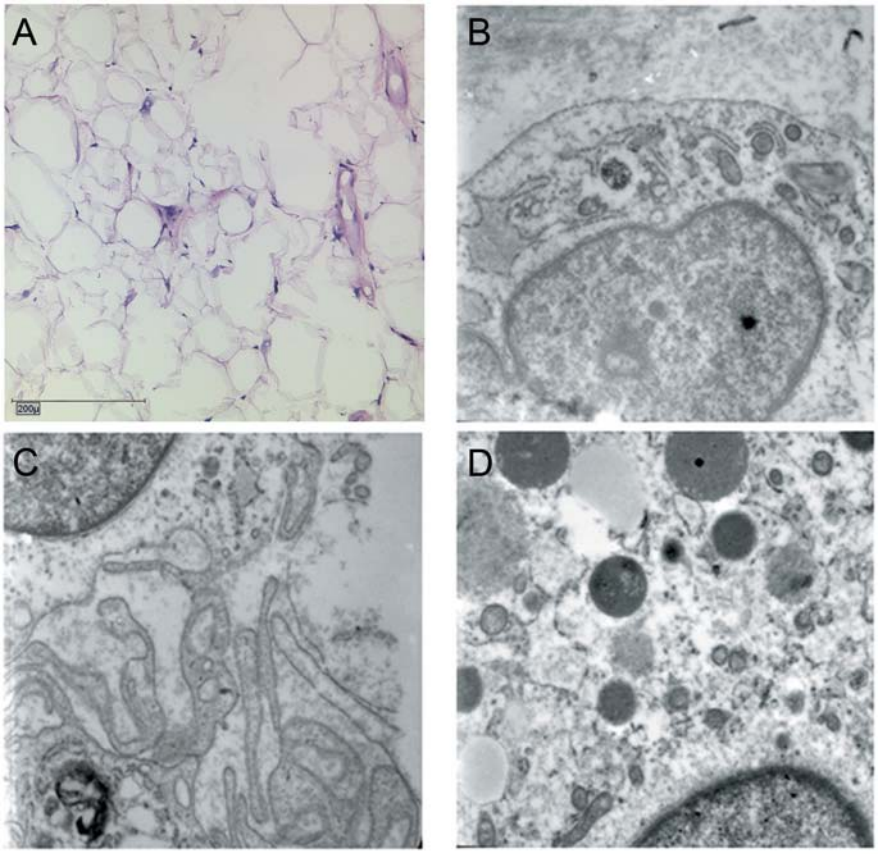

Figure 2. *Histological (a) and electron microscopic (b-d) (×12,000) morphology of the primary tumour: A: Haematoxylin and eosin staining; B: Nuclear morphology demonstrates nuclear polymorphism, with heterochromic prominent nucleoli; oval-shaped nuclei with great variation in size are apparent; C: Cytoplasm: few reticular endoplasmic reticule are apparent; D: Light and dark lipid vacuoles can be seen.*

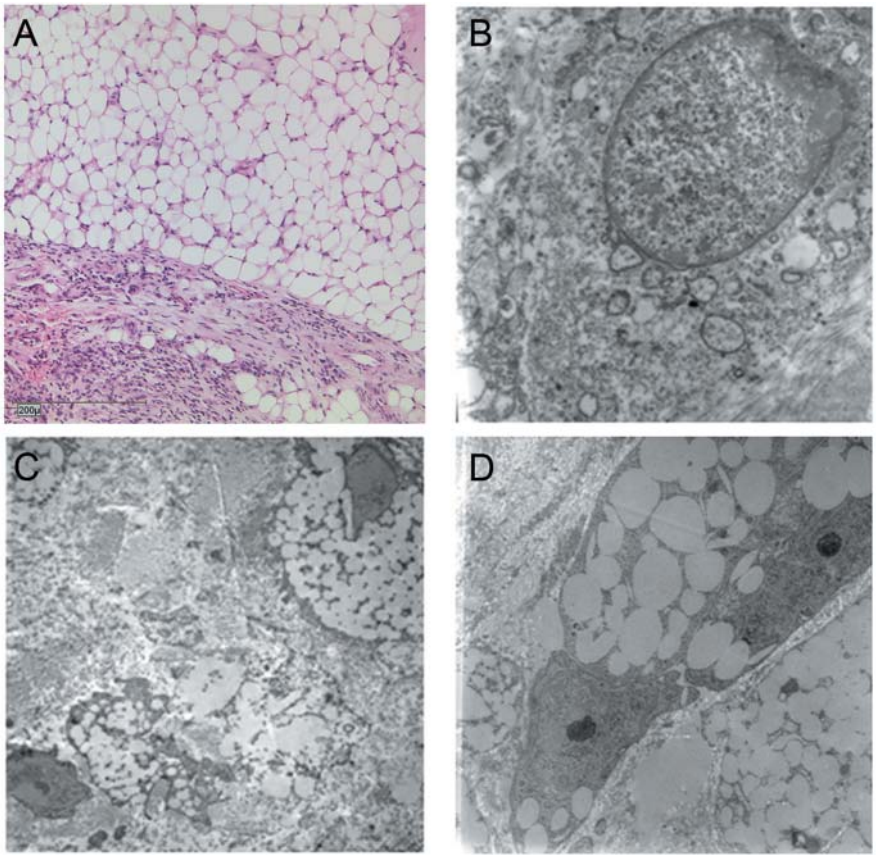

Figure 3. *Histological (A) and electron microscopic morphology (B-D) (× 12,000) of the xenograft: A: Haematoxylin and eosin staining; B: Nuclear morphology with heterochromic prominent elongated oval nucleoli; C: Lipid droplets are present; there are few signs of cell necrosis; D: Light and dark lipid vacuoles can be seen.*

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