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In vitro evaluation of a biomaterial-based anticancer drug delivery system as an alternative to conventional post-surgery bone cancer treatment

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ABSTRACT

Patients diagnosed with osteosarcoma are currently treated with intravenous injections of anticancer agents after tumor resection. However, due to remaining neoplastic cells at the site of tumor removal, cancer recurrence often occurs. Successful bone regeneration combined with the control of residual cancer cells presents a challenge for tissue engineering. Cyclodextrins loaded with chemotherapeutic drugs reversibly release the drugs over time. Hydroxyapatite bone biomaterials coated with doxorubicin-loaded cyclodextrin should release the drug with time after implantation directly at the original tumor site and may be a way to eliminate residual neoplastic cells. In the present study, we have carried out *in vitro* studies to evaluate such a drug-delivery system and have shown that doxorubicin released from cyclodextrin-coated hydroxyapatite retained biological activity and exhibited longer and higher cytotoxic effects on both cancer (osteosarcoma cells) and healthy cells (primary osteoblasts and endothelial cells) compared to biomaterials without cyclodextrin loaded with doxorubicin. Furthermore, doxorubicin released from biomaterials with cyclodextrin moderately induced the expression of tumor suppressor protein p53 whereas p21 expression was similar to control cells. In addition, hypoxic conditions, which occur after implantation until blood-flow to the area is regenerated, protected endothelial cells and primary osteoblasts from doxorubicin-induced cytotoxicity. This chemo-protective effect was far less prominent for the osteosarcoma cells. These findings indicate that a hydroxyapatite-cyclodextrin-doxorubicin chemotherapeutic strategy may enhance the drug-targeting effect on tumor cells while protecting the more sensitive healthy cells for a period of time after implantation. A successful integration of such a drug delivery system might allow healthy cells to initially survive during the doxorubicin exposure period, while eliminating residual neoplastic cells.

1. Introduction

Bone cancer can affect any parts of the skeleton by invasion (primary) or metastasis (secondary) of tumor cells and cause morbidity with severe functional and structural defects, leading to death. Surgery is the most frequent treatment of bone cancer although cancer recurrence can occur due to incomplete tumor removal [1,2]. Among malignant tumors in bone, the most common primary bone cancer is osteosarcoma (OSA) [3]. Tumor surgery together with chemo- and radiotherapy is widely used for the treatment of OSA. After tumor removal, the major challenges are (i) the reconstruction of the defect in order to maintain structural and functional activity and address aesthetic considerations and (ii) the prevention of cancer recurrence. For bone reconstruction, hydroxyapatite (HA) has been extensively examined during the last decades and is widely used as a bone substitute. The slow resorption of the material, the promotion of new bone formation combined with the similarity in composition to the mineral phase of natural bone makes HA a highly suitable material for bone replacement in clinical applications [4].

Cancer recurrence due to residual cancer cells after tumor resection is a major problem. The efficacy of post-operative chemo- or radiotherapy is uncertain and often accompanied by adverse effects that severely limit the patients' quality of life. Therefore, an effort has been made to extend the efficacy and usefulness of chemotherapy by using high-dose chemotherapy with or without autologous peripheral blood stem cell transplantation and tumor-targeted drug delivery systems [5,

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6]. However, these methods do not always show significant advantages over standard chemotherapy [7]. Therefore, a localized chemotherapeutic strategy as an alternative to overcome the major disadvantages of systemically administered chemotherapy, including toxic effects on healthy cells, has attracted much attention [8]. The application of high local chemotherapeutic concentrations may effectively kill residual neoplastic cells remaining after tumor removal. In addition, the elimination of tumor cells might be enhanced due to sustained exposure to chemotherapeutic agents throughout the cell cycle [9]. Implantable drug delivery systems, in which anticancer agents are combined with bone substitutes, have been developed. The goal is to protect the anticancer agent from a rapid *in vivo* metabolism during circulation while simultaneously delivering lower doses but increasing therapeutic effectiveness by deploying the drug at the site where it is needed over a period of time [10,11].

Different synthetic polymers such as poly(lactic-*co*-glycolic acid) have been shown to be effective as drug carriers [12]. Cyclodextrins (CD) have recently been shown to bind and release chemotherapeutic drugs when combined with a bioceramic bone graft [13]. Cyclodextrin molecules exhibit a hydrophobic character, which enables the reversible formation of inclusion complexes with many compounds such as the anthracycline doxorubicin (DOX) as well as others such as cisplatin or gentamicin [13,14]. According to the studies by Duchene et al. and Wouessidjewe et al. the loading of hydrophilic DOX occurs by adsorption [15,16].

The site of bone resection is initially an area with a low oxygen tension (hypoxia) due to lack of a functioning vasculature. Hypoxia, on the one hand, induces ingrowth of blood vessels into regenerating bone, however, on the other hand, hypoxia promotes the invasion and metastasis of cancer cells [17]. Cellular hypoxia is an incidence in normal physiology and pathological conditions such as cancer [18]. In rapidly proliferating malignant tumors, hypoxic conditions are common due to the abnormal tumor vasculature, characterized by leaky and fragile blood vessels. This is one of the main reasons that cancerous tissue often contains vast areas with low oxygen concentrations [19,20]. Tumor hypoxia is known to promote cancer invasion and metastasis, thus promoting the spread of cancer cells. In addition, a systemically administered chemotherapeutic agent has difficulty in reaching the cancer cells, as the tumor area is surrounded by leaky and fragile blood vessels [17]. In addition, tumor resection results in a large avascular area which generates a local hypoxic environment.

DNA damage can occur due to various reasons. The application of chemotherapeutic agents that target DNA can be a great problem. Several mechanisms are initiated to suppress the proliferation of cells with abnormal DNA that might give rise to cancer [21]. p53 is a transcription factor intimately involved in DNA repair, cell cycle arrest and apoptosis and the removal of cancer cells [22,23]. The cell cycle CDK (cycling-dependent kinase) inhibitor p21 is transcriptionally targeted by p53 and induces cell cycle arrest in order to prevent the passing on of damaged DNA to daughter cells that might give rise to carcinogenesis [24].

The main goal of this study was to determine whether doxorubicin released after being added to CD-HA retained biological activity and if differences in the cytotoxicity were observed when doxorubicin was added to HA alone. The aim was to evaluate the biological activity of the CD-doxorubicin-HA-based drug-delivery bone tissue scaffold system on osteosarcoma cancer cells (MG-63) compared to primary healthy cells. The cytotoxic effects of DOX were evaluated by examining the survival and growth of MG-63 compared to primary human osteoblast cells (pOBs), as well as endothelial cells (human umbilical vein endothelial cells, HUVECs, were used as models for primary endothelial cells) which form the vasculature and are required for successful bone regeneration under normoxic and hypoxic conditions. Primary cells as well as MG-63 were exposed to DOX either by direct application to or *via* leaching from a hydroxyapatite-based biomaterial with or without coating with the drug delivery component cyclodextrin (HACD or HA, respectively) and analyzed for cell viability or tube formation in endothelial cells. Beyond this, the expression patterns of the tumor suppressors p53 and p21 were determined [23]. Both molecules can serve as readout parameters of efficacy of anticancer therapy. In addition, the effect of DOX on the cell viability of the three cell types under hypoxia, which mimics the physiological conditions after surgery and during cancer therapy was analyzed.

2. Materials and methods

All cells were obtained from excess tissue and were used only after obtaining informed consent. The procedures were approved by the responsible Ethics Commission of the State of Rhineland-Palatinate, Germany.

2.1. Compounds

Doxorubicin was obtained from Hexal AG, Holzkirchen, Germany. Fetal calf serum (FCS), basic fibroblast growth factor (bFGF), sodium heparin, gelatin, medium 199, medium 199 (10×), DMEM medium, $NaHCO_3$, HEPES and bovine serum albumin (BSA) were purchased from Sigma Aldrich, St. Louis, USA. Penicillin, streptomycin, GlutaMax™, phosphate buffered saline (PBS) and Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12) were obtained from Gibco, Carlsbad, USA. Collagenase type I was purchased from Worthington, Lakewood, USA. Endothelial cell growth factor (ECGS) was from BD, Franklin Lakes, USA. Endothelial Cell Basal Medium MV was obtained from PromoCell, Heidelberg, Germany. Crystal violet was obtained from Merck, Darmstadt, Germany. NaOH was purchased from Roth, Karlsruhe, Germany.

2.2. Biomaterial and cyclodextrin-modification

The synthesis of the HA (Osbone®, Curasan AG, Kleinostheim, Germany), the coating with polycyclodextrin, the characterization of the materials and the loading with chemotherapeutic compounds has been previously described [4,13]. In brief, HA particles with a size of 20 µm were mixed with ammonium hydrogen carbonate particles of different sizes (varying from 50 µm up to 500 µm). After swaging and drying at 80 °C, HA granules with an overall porosity of 65% with a pore size of 100 and 250 µm in a ratio of 1:1 developed which mimics a structure similar to bone. A phase purity of 99% was determined by X-ray powder diffractometry [4,13]. HA granules with an average size of 2–3 mm were sintered at 1000 °C and used during this study. Subsequently, the material was coated with polycyclodextrin (Université Lille Nord de France, Lille, France). The HA granules were soaked in and impregnated with a mixture of water and β-cyclodextrin, citric acid and sodium hypophosphite and finally dried at 90 °C to finish the process. Successful incorporation of polycyclodextrin was assessed by thermogravimetric analyses [13].

2.3. Endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated from the vein of human umbilical cord according to a previously published method [25]. In brief, HUVECs were obtained by collagenase digestion (collagenase type I). The cells were expanded in medium 199 containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 0.34% GlutaMax™ and supplemented with 50 ng/ml endothelial cell growth factor (ECGS) and 50 ng/ml sodium heparin. The cells were seeded in cell culture flasks coated with 0.2% gelatin and used for experimental purposes up to passage 4. HUVECs were cultivated in Endothelial Cell Basal Medium MV, supplemented with 15% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 ng/ml basic fibroblast growth factor (bFGF) and 10 µg/ml sodium heparin 24 h prior to use in experiments.