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Tumor-targeted suicide gene-directed enzyme prodrug therapy mediated by extracellular vesicles

Minireview

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In this article, we describe the gene-directed enzyme prodrug therapy, also known as the "Trojan Horse" therapy mediated by exosomes – small extracellular vesicles (sEVs) secreted from mesenchymal stem/stromal cells (MSCs) and cancer cells. MSC-EVs possess strong migrating tropism toward tumor sites. EVs derived from tumor cells mimic the parental cells in an invasive metastatic growth trait and the capability to reprogram the recipient cells. The behavior of these EVs when modified with the suicide gene predestinates them to be a drug with guided intracellular action. EVs with therapeutic suicide gene are prepared from cells with integrated retrovirus vector containing its genetic message. These EVs are internalized by tumor cells and the product of the gene converts the non-toxic prodrug into a cytotoxic drug inside the cell causing its suicide. The action of two suicide gene systems are described: the *yCD::UPRT*-MSC/5-FC system and the HSVTK-MSC-GCV system. Suicide gene EVs either MSCs or tumor cell origin due to their intrinsic targeting capabilities, high modification flexibility, as well as biological barrier permeability represent potential drugs for tumors untreatable with present standard cancer therapies.

Key words: mesenchymal stem/stromal cell; retrovirus transduced cells; extracellular vesicles; suicide genes; "Trojan Horse" cancer therapy

The median survival time of patients with aggressive tumors such as pancreatic cancer, glioblastoma, and malignant metastatic tumors is extremely low. The inefficiency of standard classical cancer therapies for these tumors lies in their failure to target specifically malignant tumor cells, in the severe side effects caused by the drugs, and in the resistance of the tumor cells to the chemical agents. In addition, the high heterogeneity of cells in solid tumors, the presence of stromal cells, and their influence on the tumor behavior even during the course of treatment hamper the efficacy of drug therapies. To achieve further progress in the therapy of aggressive tumors and metastases an innovative therapeutic approach is obviously required. The tumor-targeting character of mesenchymal stem/stromal cells (MSCs) and their extracellular vesicles (EVs) predestine them for innovative kinds of anti-cancer therapy [1]. The character of MSC-EVs such as biocompatibility, negligible immunogenicity, stability during prolonged circulation time, ability to cross BBB, and drug loading capacity predetermine them to be prospective anticancer drugs [2, 3]. The novel cancer therapy to be curative should be targeted not only to tumor cells but also to cells of the tumor environment, especially to cancer-associated fibroblasts (CAFs) [4]. CAFs play a major role in the progression of difficult-treated malignancies due to the secretion of a wide repertoire of factors that regulate tumor progression, metastasis, and recurrence [5]. Innovative cancer drug should be able to be internalized by both tumor-initiating stem cells, tumor cells, and act intracellularly thus overcoming the natural or acquired drug resistance of tumor cells [6]. Disruption of the tumor environment destroying CAFs in a similar way may improve the drug access to tumor cells and possibly inhibit the formation of metastases or even prevent metastasis. EVs are natural mechanisms for intercellular

communication, allowing the transport of proteins, genetic material, and many other biologically active compounds to fulfill these requirements. It is becoming increasingly clear that EVs from MSCs, tumor stromal cells, and from cancer cells modified with gene are able to convert intracellularly a non-toxic drug to a potent cancer chemotherapeutic. This "Trojan Horse" strategy is a promising approach for novel cancer therapy [7]. Moreover, the old-known function of EVs as a cell waste box facilitates the production of EVs possessing mRNA of overexpressed genes in their cargo [8]. According to the updated guidelines of the International Society for Extracellular Vesicles (ISEV), EVs even produced from genetically modified MSCs are regarded as biological medicinal products [9]. According to ISEV, it is recommended to name exosomes as EVs and define them by size and cell of origin [10]. In this paper, the term sEVs is substituted by the earlier used term exosomes.

In this review, we summarize the efforts to exploit the homing ability of exosomes – EVs from MSCs and from tumor cells for the development of suicide gene-directed enzyme prodrug therapy (GDEPT), therapy also known as the "Trojan Horse" therapy. A schematic representation of steps leading to the creation of suicide gene sEVs both from MSCs and tumor cells together with forthcoming therapeutic consequences is presented in Figure 1.

MSCs secrete extracellular vesicles – exosomes

The physiological role of MSCs is to repair damaged or used tissues in the body. The cells possess the ability to migrate to the site of injury. MSCs cultivated *in vitro* secrete extracellular vesicles (EVs) into a conditioned medium (CM). Evidence suggested that MSCs mediate the repairs through EVs that stimulate endogenous repair mechanisms in a paracrine fashion. They deliver their cargoes intercellularly to distant cells. MSC-derived EVs could mimic the biological activity of their parental cells. Therefore, MSC-derived EVs can be a cell-free cancer treatment alternative. Exosomes – small EVs are round cell-derived membrane nanoparticles 30–150 nm in size enclosing proteins, s-RNAs mi-RNA, lipids, growth factors, and cytokines, the compounds that reflect the kind and character of the original cell.

The tumor, being a wound that does not heal [11], attracts MSCs and together with other cells, they form the tumor stroma. In addition, MSCs in the tumor environment under the influence of tumor cell releasing EVs are converted to CAFs, which secrete CAF-EVs as well. All properties of these MSC-EVs, when properly modified either with the genetic message or cancer drug may be tumor-tropic. Therefore, they have a potential to serve as an innovative anti-cancer drug via inhibiting tumor cell growth or inducing apoptotic cell death.

Modification of MSCs for tumor therapeutic purpose

The therapeutic capacity of MSCs can be enhanced by genetic modification leading to the expression of genes in favor of their therapeutic use. Recently, a systematic review reported the use of engineered MSCs for the treatment of various diseases. The efficacy of engineered MSCs is depen-

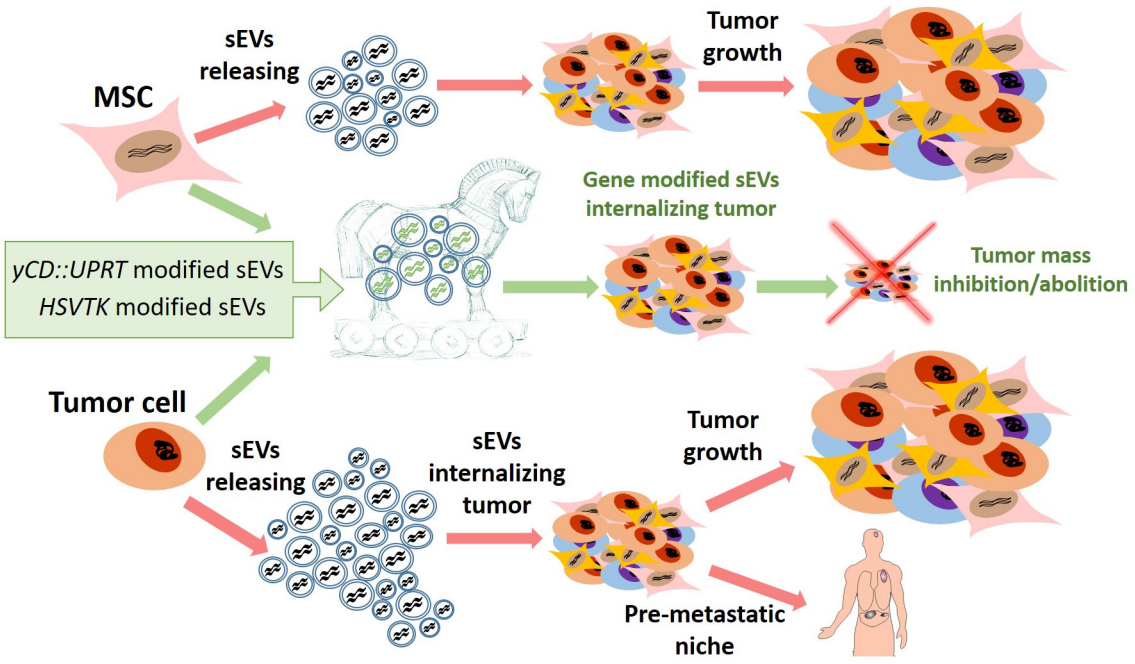

Figure 1. Schematic diagram illustrating suicide gene EVs derived from MSCs and tumor cells as an intracellularly targeted anti-cancer drug

dent on the expression of paracrine factors responsible for the therapeutic efficacy [12]. Our approach to preparing MSCs engineered to express genes capable of attacking tumor cells intracellularly was based on our experience with suicide gene therapy mediated by retroviruses [13]. The mode of retrovirus replication through the integration of its genome into cell DNA as a DNA provirus can be used for a foreign gene insertion into cells. Such a gene is expressed due to the potent retrovirus promoters, and the message is translated into a corresponding protein. This was our working approach for the development of gene-directed enzyme prodrug therapy (GDEPT) mediated by MSCs. The principle of this therapeutic arrangement is to convert a nontoxic compound, a prodrug, by enzymatic conversion into a cytotoxic drug within the tumor cell. The gene, from which the enzyme is transcribed, is called the suicide gene. The drug formed inside the cell causes its death (suicide), and the drug being a small molecular weight compound diffuses into other cells, causing a neighboring killing effect. Several GDEPT systems have been described most of them using microbial enzymes [14]. Generally, the choice of an enzyme and the availability of a suitable prodrug are the major factors determining the success and therapeutic utility of GDEPT. Enzymes from nonhuman sources, bacterial cytosine deaminase [15], nitroreductase [16], carboxypeptidase [17], purine nucleoside phosphorylase [18], or yeast origin are employed to avoid off-target toxicity. Our efforts were concentrated to use MSCs as tumor-targeted cells for the development of two prodrug suicide gene therapy systems, namely the *yCD::UPRT*-MSC/5-FC system [19] and the HSVTK-MSC/GCV system [45]. Later on both cellular systems, we found to be mediated by sEVs [21, 22].

GDEPT via *yCD::UPRT*-MSC/5-FC system

For the *yCD::UPRT*-MSC/5-FC system, we picked out the fused yeast gene cytosine deaminase::uracil phosphoribosyl transferase (*yCD::UPRT*) known for 100-times higher efficiency than bacterial cytosine deaminase [23]. The retrovirus vector was constructed as a bicistronic construct with the suicide gene separated by the internal ribosome entry site (*IRES*) sequence from the neo gene [24, 25]. Such an arrangement allows the expression of both linked genes and their translation into proteins. The cell selection with a pretested concentration of G418 antibiotic leads to a homogenous population of transduced cells. The homogeneity of cells with integrated *yCD::UPRT* retrovirus can be easily checked by the addition of the nontoxic prodrug 5-fluorocytosine (5-FC), which causes the apoptotic death of all cells due to the conversion of 5-FC to cytotoxic 5-fluorouracil (5-FU) inside the cells. The infection of any cells with a replication-defective mixed ecotropic and amphotropic envelope retrovirus containing the *yCD::UPRT* suicide gene results in transduced cells. The *yCD::UPRT* gene is expressed and translated to cytosine deaminase enzyme capable to deaminate prodrug 5-FC to cytotoxic compound 5-FU. *UPRT,* a part of the fused gene, catalyzes the phosphorylation of 5-FU to 5-FU monophosphate. Its active metabolites can inhibit DNA and RNA synthesis, leading to cell death.

Therapeutic achievements with MSCs engineered to express the *yCD::UPRT* gene

Genetically engineered MSCs have been successfully used in various animal models of the diseases. In a pilot preclinical study with nude mice, we have demonstrated that the human adipose tissue-derived MSCs engineered to express the *yCD::UPRT* gene (*yCD::UPRT*-AT-MSCs) administered intravenously were effective in significantly inhibiting subcutaneous xenografts of human colorectal carcinoma cells [19], melanoma [20], and human bone metastatic prostate cells [26]. The positive therapeutic effect of the human *yCD::UPRT*-AT-MSCs cells was proven in the autochthonous prostate adenocarcinoma in TRAMP mice, which spontaneously develop aggressive prostate cancer [27]. Intracranial administration of the *yCD::UPRT*-AT-MSCs has been shown in a preclinical study to be effective in the treatment of intracerebral rat C6 glioblastoma leading to complete tumor regression in a significant number of animals [28–30].

EVs secreted from *yCD::UPRT*-MSCs are involved in the anti-cancer effect

MSC-produced EVs have been tested as part of experimental cell-free therapies for various diseases including cancer by several authors. Comprehensive coverage of the role of MSC-EVs in tumor biology was recently reported [31]. Increasing evidence supports the notion that the mechanism of interaction between MSCs and human tumor cells involves the exchange of biological material through EVs (reviewed in [32]). Previous studies revealed that MSCs could either support or suppress tumor progression in different cancers by paracrine signaling via MSC-derived EVs. Evidence suggested that the MSC-derived EVs could mimic their parental cells, possessing the pro-tumor and anti-tumor effects and inherent tumor tropism [33]. The high therapeutic potential of human *yCD::UPRT*-AT-MSCs injected intravenously was not compatible with the later observation of bio-distribution of intravenously administered cells. Studies of bio-distribution, migration, and homing of systematically applied MSCs revealed that 80% of cells are immediately entrapped in the lung tissue and then cleared to the liver within 1 day [34]. The first circumstantial evidence of paracrine/endocrine action of *yCD-UPRT*-MSCs was observed when intravenously administered cells in tumor-bearing nude mice were difficult to find in tumors, but the therapeutic effect has been observed [19, 20, 26]. Examination of tumor cell growth-inhibiting activity of a conditional medium (CM) from

yCD::UPPRT-MSCs by various methodical approaches revealed the presence of sEVs that exhibited a growth-inhibiting effect on human tumor cells. The analysis of *yCD::UPRT*-MSC-CM by the size exclusion chromatography together with NanoSight size measurement localized the tumor cell growth-inhibiting activity in sEVs fractions. RT-PCR analysis confirmed the presence of mRNA of the *yCD::UPRT* gene in these nano-sized particles. Therefore, MSCs with cell DNA integrated retrovirus vector continuously release EVs possessing mRNA of the *yCD::UPRT* suicide gene. The *yCD::UPRT*-MSC-EVs internalized by recipient tumor cells in the presence of the prodrug 5-FC effectively triggered dose-dependent tumor cell death by endocytosed EVs via an intracellular conversion of the prodrug to 5-FU. MSC suicide gene exosomes represent a new class of tumor cell-targeting drug acting intracellularly with the curative potential [21].

Suicide gene MSC-EVs can be enriched with iron oxide nanoparticles

The iron oxide nanoparticles are useful tools for MSCs labeling. Labeled cells do not differ in cell proliferation, survival, or tumor tropism compared to parental MSCs [35]. MSCs isolated from various tissues and *yCD::UPRT*-MSCs were found to be feasible for labeling with Venofer, an iron oxide carbohydrate nanoparticle, a drug indicated for the treatment of anemia. We proved that all Venofer-labeled *yCD::UPRT*-MSCs released EVs – exosomes possessing iron oxide. These EVs were efficiently endocytosed by tumor cells and in the presence of the prodrug 5-FC inhibited tumor growth in a dose-dependent manner. The treated tumor cells were also effectively ablated following the induction of hyperthermia using an external alternating magnetic field [36].

Brain glioblastoma homing of *yCD::UPRT*-MSC-EVs

Labeling of MSCs with iron oxide nanoparticles revealed the release of exosomes possessing the iron oxide label in the exosome's cargo. When iron oxide MSCs were administered intranasally to glioblastoma-bearing rats, the label was found in brain glioblastoma supporting the evidence for tumor tropism of the MSC-EVs [37]. This observation was confirmed in a preclinical study in rats. CM containing *yCD::UPRT*-MSC-EVs repeatedly injected intraperitoneally, subcutaneously, or applied intranasally inhibited the growth of cerebral C6 glioblastomas in rats. CM from dental pulp engineered to express the *yCD::UPRT* gene was found quite effective in the C6 glioblastoma cells' eradication when applied intranasally [38]. MSCs isolated from dental pulp tissue were shown to be different from MSCs from adipose tissue or MSCs from the umbilical cord in gene expression of pluripotent stem cell genes. The neurotropism of dental pulp derive MSCs may reflect their embryonic stem cell origin from ecto-mesenchymal elements, containing neural crest-derived cells [39].

Anti-cancer drugs can be incorporated into MSC-EVs

Anti-cancer drugs loaded into MSCs are released from them in the form of EVs. They gain the cancer-homing ability and in a dependence of MSC-tissue origin, the drug delivery can be targeted specifically to tumor or metastases [40]. EVs-drug delivery may result in a reduction of severe adverse side effects. It was reported that the exposition of mouse bone marrow-derived MSCs to a very high paclitaxel (PTX) dosage *in vitro* for 24 h led to the release of EVs containing the drug in its cargo [41]. The same PTX pre-loading technique in human umbilical cord-derived MSCs lead to a similar observation. The PTX-loaded EVs exhibited tumor growth and metastases inhibitory effects in various cancer cell lines *in vitro* [42]. Similarly, human gingival papilla MSCs were primed with a high PTX concentration. The loaded PTX-EVs inhibited the growth of pancreatic carcinoma and squamous carcinoma cells [43]. In our experiments, the cultivation of human dental pulp MSCs (DP-MSCs) with gemcitabine (GCB) led to its absorption into the cells and subsequent secretion of DP-MSC-GCB-EVs. The growth inhibition activity of these EVs tested in pancreatic cell lines PANC1 and MiaPaca *in vitro* was higher compared to GCB alone. Similarly, the *yCD::UPRT*-DP-MSCs incorporated gemcitabine. Secreted EVs possessing GCB in their cargo acted as EVs with dual tumor cell inhibiting activity [44].

GDEPT via HSVTK-MSC/GCV system

The field of suicide gene therapy started in 1986 by a study where thymidine kinase of herpes simplex virus (HSVTK) was stably introduced to tumor cells as a tool for controlling tumor cell chemosensitivity [45]. Tumor tropism of MSCs led us to transduction of adipose tissue-derived human MSCs with retrovirus vector containing HSVTK gene. We found the HSVTK-MSCs could exert a cytotoxic effect on human glioblastoma cell lines upon treatment with prodrug ganciclovir (GCV). The formation of gap junctions between HSVTK-MSCs increased glioblastoma cell death by bystander cytotoxicity [46]. Infection of MSCs with a replication-defective retrovirus containing the genetic information of a suicide gene is leading to its expression from integrated DNA provirus. We found previously that such cells excrete EVs possessing suicide gene mRNA in their cargo [21]. It was not a surprise to find that the homogenous population of HSVTK-MSCs release EVs having mRNA of the suicide gene in their cargo. The HSVTK-MSC-EVs were found to be easily internalized by the tumor cells, and the presence of prodrug GCV caused their death in a dose-dependent manner. They efficiently killed both glioma cell lines and primary human glioblas-

toma cells *in vitro*. HSVTK-MSC-EVs represent a tumor intracellularly acting drug with curative potential [22].

Tumor cell-secreted EVs can be modified to anti-tumor drugs

The EVs excreted from tumor cells possess many diverse biological functions. Composition analysis and biogenesis of tumor cell-derived EVs revealed that EVs can support neoplastic growth, invasion, metastasis, and reprogram recipient cells. They play an important role in the organotropism of metastases through pre-metastatic niches formation [47]. Integrins of tumor EVs determine organotropic metastasis [48]. Tumor cell-derived EVs mimic the contents of the parent cell suggesting that EVs therapeutically modified will be bio-distributed preferentially to tumors [49, 50]. It was demonstrated that the circulating breast-cancer-derived EVs loaded with doxorubicin can mingle with their original EVs and inhibit breast cancer metastasis to the lungs [51]. We tested this hypothesis and found that cancer cells when transduced with the *yCD::UPRT* gene secreted EVs acting similarly to *yCD::UPRT*-MSC EVs. Analyses of different types of human tumor cell lines transduced with the *yCD::UPRT* gene revealed their tumor tropism. The tumor cell-derived suicide gene EVs home to their cells of origin and also to other tumor cells inhibiting the growth of tumor cells at different levels. In agreement with others [52], our data from experiments *in vitro* suggest that tumor cell-derived suicide gene-EVs can act as cancer metastatic cell-targeted drug. They generate cancer drug inside cancer cells similarly to *yCD::UPRT*-MSC-EVs [53]. Experiments in immunodeficient mice with specimens of primary human tumors could be the next approach.

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