Engineered human mesenchymal stem cells for neuroblastoma therapeutics

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Abstract. Drug-resistant neuroblastoma remains a major challenge in paediatric oncology and novel and less toxic therapeutic approaches are urgently needed to improve survival and reduce the side effects of traditional therapeutic interventions. Mesenchymal stem cells (MSCs) are an attractive candidate for cell and gene therapy since they are recruited by and able to infiltrate tumours. This feature has been exploited by creating genetically modified MSCs that are able to combat cancer by delivering therapeutic molecules. Whether neuroblastomas attract systemically delivered MSCs is still controversial. We investigated whether MSCs engineered to express tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) could: i) cause death of classic and primary neuroblastoma cell lines in vitro; ii) migrate to tumour sites in vivo; and iii) reduce neuroblastoma growth in xenotransplantation experiments. We observed that classic and primary neuroblastoma cell lines expressing death receptors could be killed by TRAIL-loaded MSCs in vitro. When injected in the peritoneum of neuroblastoma-bearing mice, TRAIL-MSCs migrated to tumour sites, but were unable to change the course of cancer development. These results indicated that MSCs have the potential to be used to deliver drugs in neuroblastoma patients, but more effective biopharmaceuticals should be used instead of TRAIL.

Introduction

Neuroblastoma, one of the most aggressive extracranial solid tumours occurring in childhood, remains a major cause of cancer-related deaths in infancy (1). Despite therapeutic strategies based on chemotherapy, radiotherapy, surgery, GD2-targeted immunotherapy, stem cell transplant and treatment with 13-cis-retinoic acid, high-risk neuroblastoma outcome remains poor, with a 5-year event-free survival <40% (2-5). Tumours show initial response to therapeutic interventions but typically relapse into an incurable form of the disease. Moreover, several drugs cause severe side effects, including cognitive impairment and retarded growth (6). Thus, to reduce drug toxicity and to improve the outcome and the lifestyle of the patients affected by neuroblastoma, additional therapeutic options are required.

Recently, cell-based approaches have been increasingly investigated for the delivery of therapeutics agents. Mesenchymal stem cells are multipotent adult stem cells isolated from the umbilical cord, bone marrow and fat tissue and can differentiate into multiple tissues including bone, cartilage, muscle, fat cells and connective tissue (7-12). Bone marrow-derived mesenchymal stem cells (MSCs) are ideally suited for the delivery of anticancer agents to tumours, including cytokines, interferons and prodrugs (13-15). MSCs are particularly suitable for the role of vectors for anticancer therapies for various reasons (16). MSCs are immunologically inert due to their low expression of constitutive major histocompatibility complex 1 (MHC1) and lack of MHC2 and co-stimulatory molecules CD80, CD86 and CD40, meaning that allogeneic cells can be used in immunocompetent patients abrogating the need of immunosuppressive therapies (17). Furthermore, MSCs are able to migrate to and incorporate into the tumour stroma when administered in vivo and, if engineered with viral vectors, can deliver therapeutic molecules that inhibit tumours or metastatic growth (18,19).

In recent studies, MSCs have been engineered to express IFN-γ, IL-12, IL-24 and tumour necrosis factor-related
apoptosis inducing ligand (TRAIL), to induce death of tumour cells (15,20).

TRAIL is a member of the TNF superfamily and interacts with fully functional death receptors DR4 and DR5, decoy receptors DCR1 and DCR2, and osteoprotegerin (OPG), which lack functional cytoplasmic signalling domains (21-23). TRAIL is an interesting anticancer molecule, since it causes apoptosis of cancer cells bearing DR4 and DR5 death receptors, but it is unable to harm normal cells which express high levels of TRAIL decoy receptors and low levels of TRAIL death receptors on their surface. TRAIL-mediated killing is not dependent on a specific molecular alteration and all molecular subtypes or high-risk tumours are potentially amenable to TRAIL killing. Thus, from a clinical point of view, TRAIL-based therapies are especially attractive due to the extremely high therapeutic index.

Clinical trials in which cancer patients have been treated with soluble, truncated forms of TRAIL have been unsuccessful, due to the extremely short half-life of the molecule in the bloodstream and the emergence of resistance; therefore, efficient TRAIL delivery is essential (24,25). The major advantage of using MSCs to deliver TRAIL is that it is continuously produced at the tumour site, overcoming the problem of the short half-life of the protein. Furthermore, it has been revealed that the full-length TRAIL protein secreted by MSCs transduced with a lentiviral vector containing the TRAIL cDNA, can resolve resistance in lung, colorectal and breast cancer (26-28). These cells are also able to clear lung metastasis in mice injected with extremely aggressive breast cancer cells or reduce the growth of mesothelioma cells in mouse models (29).

Notably, a protease inhibitor currently used in the clinic, Bortezomib, is able to enhance TRAIL-mediated killing of neuroblastoma cells or render them sensitive to the molecule, suggesting that in a TRAIL therapy setting, the problem of resistance could be managed pharmacologically (30-32).

In the present study, we investigated the tumour-homing ability and anticancer activity of TRAIL-MSCs in the context of neuroblastoma.

Materials and methods

Cell lines. The primary human neuroblastoma cell lines A5, 2820, 0396 and 1043008 were isolated at the Institute of Child Health (University College London, London, UK) by disaggregating surgical resections. The patient characteristics are summarised in Table I. Consent for the isolation of cell lines from patient material was obtained in accordance with the Great Ormond Street Hospital (London, UK) Ethics Committee regulations.

The patient-derived xenograft (PDX) cells LU-NB-1, LU-NB-2 and LU-NB-3 were established and characterized at the laboratory of Dr Daniel Bexell (Lund University, Lund, Sweden) as previously described (33). hNB cells were isolated from a tumour metastatised in the neck of a 3-year-old male patient in 2011 (34). MSC-TRAIL was generated at the laboratory of Dr Samuel Janes (University College London, London, UK) as previously described (26).

SKNAS, IMR-32, Kelly, SHEP, LA-N-5 and SH-SY5Y were obtained from the American Type Culture Collection (ATCC; Teddington, Middlesex, UK). LA-N-1 and patient-derived neuroblastoma cell lines were provided by Dr John Anderson (Institute of Child Health). LA-N-1, SKNAS, SHEP and SH-SY5Y were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.), 2 mM glutamine (Gibco; Thermo Fisher Scientific, Inc.) and 10 mM sodium pyruvate (Gibco; Thermo Fisher Scientific, Inc.). IMR-32, Kelly, LA-N-5 were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS, 2 mM glutamine supplemented, 10 mM sodium pyruvate and 10 mM non-essential amino acids (NEAA) (Gibco; Thermo Fisher Scientific, Inc.). hNB cells were cultured in RPMI-1640 medium supplemented with 20% fetal calf serum (FCS) (Gibco; Thermo Fisher Scientific, Inc.), 2 mM glutamine, 10 μM 2-mercaptoethanol (Thermo Fisher Scientific, Inc.), 1 mM sodium pyruvate, 1% penicillin/streptomycin and 10 mM NEAA.

TRAIL-MSCs were grown in α-MEM (Gibco; Thermo Fisher Scientific, Inc.) with 16% FBS, 4 mM L-glutamine and 1% penicillin/streptomycin.

Primary cell lines were grown in stem cell medium (DMEM/F-12 medium with glutamine, 1% penicillin/streptomycin, 2% B27 supplement (Thermo Fisher Scientific, Inc.), 40 ng/ml of basic fibroblast growth factor (FGF) (PeproTech, Inc., Rocky Hill, NJ, USA) and 20 ng/ml of epidermal growth factor (EGF) (PeproTech, Inc.). All cell lines were incubated at 37°C and 5% CO₂.

Western blotting. Cells were lysed in Laemmli buffer (cat. no. NP0007; Thermo Fisher Scientific, Inc.) supplemented with β-mercaptoethanol (cat. no. 21985023; Thermo Fisher Scientific, Inc.) and protein concentrations were determined using a Pierce BCA Protein Assay kit (cat. no. 23227; Thermo Fisher Scientific, Inc.). The protein extracts (20 μg) were resolved in 10% acrylamide gel and transferred onto nitrocellulose membranes which were blocked in 5% non-fat dry milk for 1 h at room temperature. The membranes were incubated overnight at 4°C with the primary antibodies followed by incubation with the goat-anti rabbit IgG HRP-conjugated secondary antibody (dilution 1:3,000; cat. no. 1706515; Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 1 h at room temperature. Membranes were exposed to ECL Western Blotting substrate (cat. no. 32106; Thermo Fisher Scientific, Inc.) as described in the manufacturer's protocol.

The following antibodies were used: DR4 (dilution 1:1,000; cat. no. 42533), DR5 (dilution 1:1,000; cat. no. 8074), α-tubulin (dilution 1:1,000; cat. no. 2144; all were from Cell Signalling Technology, Danvers, MA, USA).
Results

Expression of DR4 and DR5 death receptors in neuroblastoma cells. The presence of death receptors in cancer cells is a valuable biomarker to determine sensitivity to TRAIL. We therefore quantified the expression of DR4 and DR5 in a panel of established (classic) or patient-derived neuroblastoma cells and investigated whether the anticancer drug Bortezomib could be used to enhance the expression of TRAIL receptors.

All classic neuroblastoma cells expressed DR5 receptor while the expression of DR4 was most prominent in a subset of neuroblastoma cell lines. The expression of DR5 was markedly increased after treatment with 20 nM Bortezomib for 24 h whereas the DR4 receptor was increased by the drug only in the Kelly and SKNAS cell lines (Fig. 1A).

There was a wide difference in expression of death receptors in patient-derived cell lines, ranging from strong to undetectable. In keeping with the results obtained with classic neuroblastoma cell lines, Bortezomib enhanced the expression of DR4-5 also in primary cells (Fig. 1B).

Flow cytometry of lentivirus-transduced cells. For the expression detection of TRAIL, MSC cells were stained with a phycocyanin (PE)-conjugated anti-TRAIL antibody (dilution: 1:100; cat. no. 550516; BD Biosciences, Franklin Lakes, NJ, USA) and analysed by flow cytometry.

Statistical analysis. All data are expressed as the means ± standard deviation (SD). Statistical significance between different test conditions was determined using Student’s t-test. Probability values <0.05 were considered to indicate a statistically significant result. The statistical analysis of survival was carried out using a log-rank test and the SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA).

Table I. Primary cell lines and patient information (Great Ormond Street Hospital cohort).

<table>
<thead>
<tr>
<th>Pathology number</th>
<th>A5</th>
<th>2820</th>
<th>1043008</th>
<th>0396</th>
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<tbody>
<tr>
<td>Procedure</td>
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<td>Diagnostic biopsy</td>
<td>Biopsy, second relapse</td>
<td>Diagnostic biopsy</td>
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<tr>
<td>Diagnosis</td>
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<td>Poorly differentiated neuroblastoma</td>
<td>Poorly differentiated neuroblastoma</td>
<td>Poorly differentiated neuroblastoma</td>
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<tr>
<td>Site of origin</td>
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<td>Abdominal mass</td>
<td>Supraclavicular lymph node</td>
<td>Right inguinal lymph node</td>
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<tr>
<td>MYCN status</td>
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<td>Not amplified</td>
<td>Diagnostic biopsy: not amplified</td>
<td>Not amplified</td>
</tr>
<tr>
<td>Chromosomal abnormalities</td>
<td>1p loss, 11q and 17q status inconclusive</td>
<td>1p loss, 11q loss, 17 gain</td>
<td>Diagnostic biopsy: 1p loss, 11q loss, 17q gain, no 1p/1q imbalance</td>
<td></td>
</tr>
<tr>
<td>Histopathology immunostaining</td>
<td>CD56+, NB84+</td>
<td>CD56+, NB84+</td>
<td>Diagnostic biopsy: CD56+, NB84+</td>
<td>CD56+, NB84+</td>
</tr>
</tbody>
</table>

Quantified by AF647-conjugated Annexin V (cat. no. A23204; Invitrogen; Thermo Fisher Scientific, Inc.) and 2 μg/ml DAPI (cat. no. D9542; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) staining using flow cytometry.

Primary patient-derived neuroblastoma cell lines were grown as a monolayer in 96-well plates coated with human recombinant laminin 521 (cat. no. LN-521; Biolamina AB, Sundbyberg, Sweden) according to the manufacturer’s instructions.

Xenograft cancer models. All experimental procedures were non-retrospectively approved by a the Brunel University London Ethics Committee and the Home Office and were conducted under the Animal Scientific Procedures Act, 1986 (UK). In addition, we confirm that the tumour burden did not exceed the recommended dimensions.

Forty-four-week-old female mice (initial weight 20-23 g), immunodeficient NOD/SCID (purchased from Charles River Laboratories, Margate, UK) were injected subcutaneously into the right flank with 5x10^6 neuroblastoma cells in a 1:1 mixture of Matrigel (Corning, Inc., Corning, NY, USA) and phosphate-buffered saline (PBS). After tumours reached the size of 5 mm in diameter, the mice were randomly assigned to 4 groups (10 mice/group) and PBS, 5x10^6 TRAIL-MSCs labelled with DiR (cat. no. D9542; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 2 µg/ml DAPI (cat. no. D12731; Thermo Fisher Scientific, Inc.) staining using flow cytometry.
different cell lines were subjected to in vitro killing assays. Neuroblastoma cells were cultured in the presence of soluble, recombinant TRAIL (r-TRAIL), Bortezomib, TRAIL-MSCs and their combinations. Before performing the experiments, TRAIL expression on the surface by the modified MSCs was verified by flow cytometry (Fig. 2A). The results revealed that soluble TRAIL and TRAIL-MSCs could induce killing of death receptor-positive cell lines that was increased by
Bortezomib. The classic neuroblastoma cell line SKNAS was particularly sensitive to the combination, with 60-70% of cells undergoing apoptosis (Fig. 2B). Primary cells were generally less sensitive to soluble TRAIL or TRAIL-MSCs, and, as anticipated, the degree of cell death was in most instances proportional to the expression levels of the TRAIL receptors (Fig. 2C). Intraperitoneally injected TRAIL-MSCs migrate to neuroblastoma xenotransplants. Since TRAIL-MSCs and the combined treatment with Bortezomib enhanced TRAIL-induced apoptosis in vitro, we evaluated the therapeutic efficacy of this treatment in vivo. SKNAS were injected subcutaneously into the flank of NOD/SCID mice to establish a xenograft tumour model.

Tumours were allowed to grow until they reached a volume of 50 mm³. Mice were then randomly assigned into 4 groups and treated with vehicle (PBS), TRAIL-MSCs labelled with the fluorescent lipophilic dye DiR, Bortezomib or their combination.

Fluorescent TRAIL-MSCs were tracked in vivo using the IVIS Lumina Imaging System (Caliper Life Sciences). Animals were imaged 24 h after injection to determine the localisation of TRAIL-MSCs in vivo. A strong signal from DiR-labeled TRAIL-MSCs was detected in the flanks containing the tumour masses whereas a weak signal was detected in other anatomical locations (Fig. 3). Control animals receiving no MSC injections were negative for fluorescence. To evaluate the effect of TRAIL-MSCs in tumour growth, mice were injected with TRAIL-MSCs, Bortezomib or TRAIL-MSCs+Bortezomib every 3 days for 3 weeks and monitored until tumour masses reached a diameter of 1.2 cm or mice lost 20% of their weight or showed signs of distress. There were no significant changes in tumour volumes in mice injected with the TRAIL-MSCs compared with the control group, but a significant anticancer effect was noted in the Bortezomib groups (Fig. 4A). We did not observe statistically significant differences in survival between different treatment groups, but there was a trend of increased survival in the Bortezomib-treated mice, consistent with previous observations (32) (Fig. 4B).

**Discussion**

Gene rearrangements and mutation of key oncogenes such as MYCN, ALK, ATRX, TERT and chromosomal losses or reduced expression of CLU, CHD5, PHOX2B, CASZ1,
PML and other tumour suppressor genes are thought to play a crucial role in the pathogenesis of neuroblastoma (35-39). Despite the significant advances in the understanding of its molecular causes, the life expectancy of patients bearing high-risk neuroblastoma is still very poor, suggesting that new therapeutic approaches are urgently needed. MYCN, the main neuroblastoma oncogene, is essentially undruggable (40). Small molecule inhibitors against ALK have been developed, however, monotherapies with small molecule inhibitors are prone to the problem of resistance and new therapies based on the inhibition of molecular drivers, for example by small interfering RNAs or CRISPR, are still far from being translated into clinical practice.

One of the many challenges of developing a therapy based on biomolecules is the delivery of these to the tumour site. It has been recently revealed that MSCs can specifically home in the tumour stroma, and this property can be exploited for cancer therapy. In this context, the death ligand TRAIL was reported to induce apoptosis selectively in tumour cells while sparing normal cells in vitro and in vivo (41,42). Recently, several research groups have explored the possibility of using TRAIL as an anticancer molecule that could be delivered in the tumour microenvironment by mesenchymal stem cells. Loebinger et al demonstrated that TRAIL-MSCs were able to home and kill tumour cells, and significantly induce cancer regression in a lung metastatic cancer model of breast cancer (20). In addition, TRAIL-expressing MSCs exhibited anticancer activity in other experimental tumour models, such as glioma and sarcoma (43-45). Despite expressing death receptors, cancer cells can become resistant to TRAIL-induced apoptosis (46,47). Several studies have indicated that Bortezomib can reverse resistance of cancer cells to TRAIL killing by increasing the expression of DR4 and DR5 (48). Notably, Naumann et al reported that Bortezomib synergised with TRAIL in inducing apoptosis of neuroblastoma cells (49).

A key question for the potential exploitation of MSCs in neuroblastoma therapeutics is whether stromal cells injected systemically can reach tumour sites. Contradicting information has been published that still leaves the question open. For example, a previous study suggested that neuroblastoma tumours are unable to attract systemically injected MSCs, whereas a recent study revealed imaging of MSCs infiltrating human neuroblastomas growing in vivo (50,51).

To further address this issue, we investigated whether MSCs engineered to express TRAIL were attracted by and able to kill neuroblastoma cells transplanted into immunocompromised mice. Firstly, MSCs expressing TRAIL were co-cultured with classical and patient-derived neuroblastoma cell lines and subjected to cell killing assays. The protease inhibitor and anticancer drug Bortezomib was used to increase the expression of death receptors and sensitize cells to TRAIL killing. Once we had identified the cell line more susceptible to TRAIL-MSCs killing, we carried out xenotransplantation experiments to assess whether the engineered stem cells were attracted by neuroblastomas. Bioluminescent imaging (BLI) clearly indicated that TRAIL-MSCs infiltrated neuroblastomas hours after intraperitoneal injections. Disappointingly, despite the fact that TRAIL-MSCs were able to kill SKNAS cells in vitro, they were not able to do so in vivo and the marginal therapeutic effect that we observed was caused by Bortezomib. Resistance to TRAIL killing may have different causes, including high expression of decoy receptors and downregulation or upregulation of apoptotic proteins. Expression of the intracellular apoptotic inhibitor c-FLIP can confer TRAIL resistance in different types of cancer cell lines and it may be involved in the protection of neuroblastoma cells from the cytotoxic effect of TRAIL (52). Deficient expression of caspases, in particular caspase-8, essential with FADD to form the death receptor complex DISC, may contribute to TRAIL resistance (53). Also, overexpression of Bcl-2 or Bcl-x or loss of Bax and Bad function or high expression of an inhibitor of apoptotic proteins could lead to TRAIL resistance (54). Furthermore, high expression of inhibitors that act downstream of the receptors, which includes XIAP, c-IAP1, c-IAP2 and survivin could block the activation and activity of caspase-9, -3 and -7 (55). It is possible that one of these mechanisms of resistance is activated and selected in tumours developing in vivo and could be responsible for the failure of TRAIL-MSCs to kill their targets. An alternative explanation of the failure of the MSCs to induce cancer regression could be that the cells have lost their ability to express TRAIL in the tumour microenvironment. Although we cannot exclude this possibility, it is unlikely since the same TRAIL-modified MSCs have been previously used to successfully inhibit breast cancer growth in immunodeficient mice (20,26).

Nonetheless, the present study suggests that mesenchymal stem cells are suitable for neuroblastoma cell and gene therapy but should be loaded with biomolecules more effective than TRAIL in further preclinical studies. For example, MSCs producing interferon gamma (IFNγ) have shown promise in reducing neuroblastoma growth when injected intratumorally (56). In light of this study, it is likely that systemic injections of MSCs producing IFNγ or other neuroblastoma-specific drugs could be successfully developed for neuroblastoma therapeutics.

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Availability of data and materials

The datasets and cell lines used in the study are available from the corresponding author upon reasonable request.

Authors' contributions

The study was conceived and written by AS and VN. VN performed all the experiments with the assistance of RP and KK. SMJ, JB, JA and DB provided the patient-derived materials, the cell lines and the key reagents, and were involved in the data analysis and the interpretation of the results. NS
carried out the pathology analyses. EK assisted with the interpretation, revision and analysis of the data. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Consent for the isolation of cell lines from patient material was obtained in accordance with the Great Ormond Street Hospital (London, UK) Ethics Committee regulations. All experimental procedures were non-retrovisibly approved by the Brunel University London Ethics Committee and the Home Office and were conducted under the Animal Scientific Procedures Act, 1986 (UK).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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