TARGETED USE OF UMBILICAL CORD MATRIX STEM CELLS FOR CANCER THERAPY

by

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B.V.Sc & A.H., A.N.G. Ranga Agricultural University, Hyderabad, India, 2001

AN ABSTRACT OF A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Anatomy and Physiology
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Abstract

Umbilical cord matrix stem (UCMS) cells are derived from Wharton's jelly and have been shown to express genes characteristic of primitive stem cells. They can be isolated in large numbers in a short time and thus potentially represent an abundant source of cells for therapeutic use. We investigated the migratory nature of human UCMS cells towards MDA 231 human breast carcinoma cells in an *in vitro* model of cell migration; UCMS cells cultured with or without MDA 231 cells for 24 hours. Next, we evaluated the effect of chemokines, stromal derived factor 1 (SDF-1) and vascular endothelial growth factor (VEGF) on human UCMS cells by treating with increasing doses of SDF-1 and VEGF. UCMS cells were found to migrate towards MDA 231 cells in a dose dependent manner. Both SDF-1 and VEGF induced migration of UCMS cells in a dose dependent manner. These results suggest that MDA 231 cells might be releasing chemokine factors, such as SDF-1 and VEGF, which promote UCMS cell migration towards the tumor cells *in vitro*. Stem cells that migrate to tumors may allow targeted delivery of therapeutic agents that otherwise may have severe side effects. To evaluate the selective engraftment and therapeutic efficiency of human UCMS cells that were engineered to express interferon beta (UCMS-IFN-β), MDA 231 cells (2×10^6) were intravenously injected into severe combined immune deficient (SCID) mice, followed by three weekly intravenous injections of fluorescently labeled UCMS-IFN-β cells (0.5×10^6). To evaluate the synergistic effect of 5-Fluorouracil (5-FU) and IFN-β, MDA 231 cells were intravenously injected into SCID mice, followed by three weekly intravenous injections of fluorescently labeled UCMS-IFN-β cells and three weekly intra peritoneal injections of 5-FU. In both of the above experiments, mice were euthanized

one week after the last UCMS cell transplant and lung weights were compared to the controls to determine the differences in tumor burden. After transplantation of UCMS-IFN-β cells into MDA 231 tumor-bearing mice, UCMS cells were found near or within metastatic lung tumors but not in other tissues, and in these animals, the lung weight was significantly less than MDA 231 tumor-bearing animals that received saline injections. Histologically, there was significant reduction in the tumor area in MDA 231 tumor bearing lungs after UCMS-IFN-β treatment. When 5-FU was given along with UCMS-IFN-β cells, there was further reduction in tumor area. These results indicate that UCMS cells can potentially be used for targeted delivery of cancer therapeutics.

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Table of Contents

LIST OF FIGURES…………………………………………………………………….....ix

LIST OF TABLES…………………………………………………………………....x

ACKOWLEDGEMENTS……………………………………………………………...xi

DEDICATION………………………………………………………………………..xiii

CHAPTER 1 - BACKGROUND AND SIGNIFICANCE 1

STEM CELLS.. 1

UMBILICAL CORD MATRIX STEM CELLS 2

TUMOR AND TUMOR MICROENVIRONMENT 4

GENE THERAPY 6

POTENTIAL ROLE OF STEM CELLS IN HOMING AND GENE THERAPY 8

CHAPTER 2 - Comparison of transduction efficiency in human UCMS cells by wild type and fiber-modified adenoviruses 26

Abstract.. 27

Introduction.. 27

Material and methods 30

Results 31

Discussion 35

CHAPTER 3 - *In vitro* migration of human umbilical cord matrix stem cell in response to chemotactic signals from cancer cells 41

Abstract.. 42

Introduction.. 42

Methods 45

Results 48

Discussion 51

CHAPTER 4 - Development of human umbilical cord matrix stem cell-based gene therapy for experimental lung tumors ... 60
Abstract ... 61
Introduction ... 62
Materials and methods ... 64
Results ... 69
Discussion ... 76

CHAPTER 5 - Combination treatment of human umbilical cord matrix stem cell-based interferon-beta gene therapy and 5-fluorouracil significantly reduces growth of metastatic human breast cancer in SCID mouse lungs ... 85
Abstract ... 86
1. Introduction ... 86
2. Materials and Methods ... 88
3. Results ... 94
4. Discussion ... 102

List of Figures

Figure 1.1 H & E stained cross section of umbilical cord and morphology of umbilical cord matrix stem cells in a culture ... 3
Figure 2.1 Adenoviral mediated expression of β-Gal. ... 32
Figure 2.2 Human UCMS cells transduced with Ad5/K4. ... 33
Figure 2.3 Human UCMS cells transduced with Ad5/K7. ... 34
Figure 2.4 Human UCMS cells transduced with Ad5/K21. ... 34
Figure 3.1 *In vitro* migration effect of UCMS cells toward MDA 231 human breast carcinoma cells. ... 49
Figure 3.2 *In vitro* migration effect of UCMS cells toward chemokines (SDF-1 and VEGF). ... 51
Figure 4.1 *In vitro* effect of human UCMS cells and human UCMS–IFN-β cells conditioned media on MDA 231 cells. ... 71
Figure 4.2 Absence of tumor formation in SCID mice injected with human UCMS cells. ... 72
Figure 4.3 Effect of human UCMS cells (not expressing IFN-β) on tumor burden. ... 73
Figure 4.4 Representative lungs of experimental groups. ... 75
Figure 4.5 Comparative Lung weights. ... 76
Figure 5.1 *In vitro* apoptotic effect of UCMS-IFN-β cell conditioned medium on MDA 231 cells. ... 96
Figure 5.2 Western blot analysis of caspase 3 activation in MDA 231 cells treated with 5-FU and the conditioned medium with UCMS-IFN-β cells. ... 97
Figure 5.3 Comparative Lung weights. ... 99
Figure 5.4 Selective engraftment and therapeutic effect of human UCMS-IFN-β cells in combination with 5-FU on MDA 231 lung tumors in SCID mice. ... 100
Figure 5.5 Combined effect of 5-FU and UCMS-IFN-β cells on tumor burden. ... 101

List of Tables

Table 1.1 Summary of viral and non viral gene delivery systems 8

Table 4.1 IFN-β (international units) secreted by $1x10^6$ human UCMS–IFN-β cells when transduced with 12500, 6400 and 3200 IFN-β adeno viralparticles/cell. 70

Table 5.1 The levels of IFN-β secreted by $1x10^6$ human IFN-β transfected UCMS cells were dose- and time-dependently increased. 95

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I would like to dedicate my thesis to my Dad, Mom, brother, my wife, all my teachers, and to all me friends for all their valuable advices and for their belief in me without which I would not have able to complete my degree

CHAPTER 1 - BACKGROUND AND SIGNIFICANCE

STEM CELLS

Stem cells are defined as unspecialized (undifferentiated) cells that have the ability to self renew and differentiate into multiple cells or tissues (Caplan, 1991). The most primitive stem cell is the fertilized oocyte. The daughter cells of the first two divisions of the fertilized egg are totipotent cells, which are able to differentiate into all three germ layers (ectoderm, mesoderm and endoderm) including extra embryonic membranes. There are two broad categories of stem cells: embryonic stem cells and adult stem cells. Embryonic stem cells (ESC) are derived from the inner cell mass of the mouse/human blastocyst, 5 days after an egg is fertilized (Evans and Kaufman, 1981). ESCs are pluripotent cells; they can differentiate into any cell/ tissue type except extra embryonic membranes (Nagy et al., 1990). ESCs express pluripotent stem cell markers, such as Oct-4, Sox-2, and Nanog and these transcription factors ensure the suppression of genes that lead to differentiation (Adewumi et al., 2007). Though ESCs were shown to differentiate into several cell types and used for replacement therapies, the major drawback of these cells is that they form tumors when transplanted in large numbers (Thomson and Marshall, 1998;Arnhold et al., 2004). There are moral/ethical concerns regarding human ESCs since they are derived from human embryos or human fetal tissues (Vats et al., 2002).

Most adult tissues have multipotent stem cells that have the property to differentiate into more than one germ layer but not all types. Adult stem cells, such as

hematopoietic stem cells and bone marrow-derived mesenchymal stem cells (MSC), also known as marrow stromal cells are harvested from adult bone marrow (Werts et al., 1980). Other sources of adult stem cells include MSCs derived from adipose tissue, umbilical cord blood, placenta, amniotic fluid, perivascular areas and from umbilical cord matrix (Campagnoli et al., 2001;Zuk et al., 2001;Mitchell et al., 2003a;Tsai et al., 2004;Sarugaser et al., 2005b). Another source of adult stem cells are neural stem cells derived from both developing and adult brain (Palmer et al., 1997). Stem cells with their unique self renewal and migratory ability in response to chemotactic factors have a great potential to be used as gene delivery agents in various autoimmune and debilitating diseases.

UMBILICAL CORD MATRIX STEM CELLS

The umbilical cord, found in amniotes is a cord that connects fetus to the placenta. The umbilical cord consists of two arteries (umbilical artery), a vein (umbilical vein), and surrounding connective tissue matrix, also called ‘Wharton’s jelly’. The umbilical cord helps in transport of nutrients and oxygen rich blood between fetus and placenta. The origin of umbilical cord is still unclear; the Wharton’s jelly contains mesenchymal like cells surrounded by extracellular matrix. The extracellular matrix contains abundant collagen and glycosaminoglycans (70% of which is hyaluronidase). During the last decade, umbilical cord blood has been extensively used for therapeutic purposes in patients with bone marrow related problems. Umbilical cord blood is a source of hematopeitic stem cells (Consolini et al., 2001;Wright-Kanuth and Smith, 2001) and mesenchymal stem cells (Kogler et al., 2004;Sanberg et al., 2005).

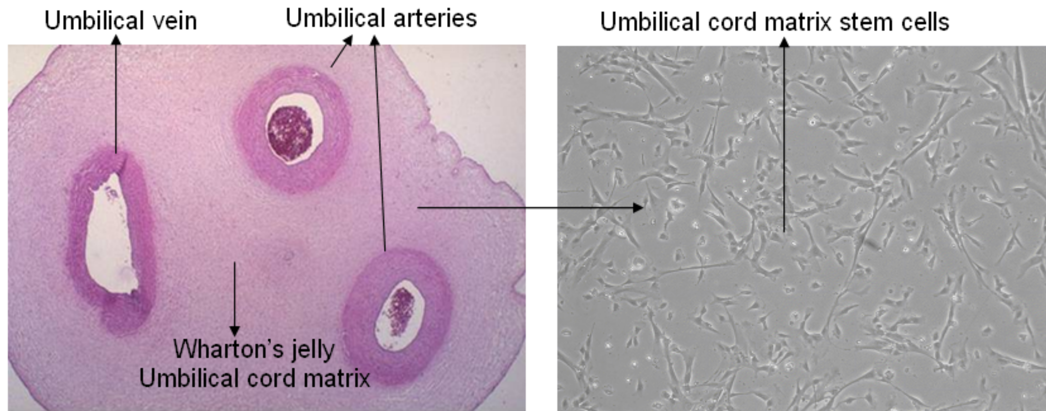

Figure 1.1 H & E stained cross section of umbilical cord and morphology of umbilical cord matrix stem cells in a culture

Multipotent stem cells called umbilical cord matrix stem (UCMS) cells are isolated from the umbilical cord matrix (Mitchell et al., 2003b). Other labs have isolated multipotent mesenchymal stem cells from various parts of umbilical cord (Kogler et al., 2004;Sarugaser et al., 2005a). UCMS cells are postnatal cells and can be harvested non-invasively in large numbers. The latter is particularly important since it has been estimated that a typical transplantation dose for human therapy is about a billion cells (Normile, 2007). UCMS cells synthesize the three major proteins associated with the pluripotent state (Sox2, Nanog, and Oct4) (Carlin et al., 2006). To test the safety of UCMS cells for transplantation studies, UCMS cells were transplanted in large numbers in SCID mice to assess possible teratoma or other tumor formation, as is sometimes the case with other primitive stem cells (Thomson and Marshall, 1998;Arnhold et al., 2004). No evidence of teratoma or other tumor was noted, indicating the cells are safe to use as drug delivery vehicles (Rachakatla et al., 2007). Recent work demonstrated that UCMS cells transduced with recombinant fiber modified adenovirus containing IFN-β

can secrete sufficient IFN-β to kill MDA 231 cells *in vitro*. In the *in vivo* studies involving MDA 231 breast carcinoma lung tumor models, UCMS cells administered systemically via the tail vein exhibited selective engraftment in the MDA 231 lung tumors of SCID mice. Tumor burden was significantly reduced following systemic administration of human IFN-β-expressing UCMS cells into tumor-bearing SCID mice (Rachakatla et al., 2007).

TUMOR AND TUMOR MICROENVIRONMENT

Tumors are sites of inflammatory cytokine and chemokine production (Hall et al., 2007). Apart from tumor cells themselves, tumors have a supportive, non tumor stroma. There are thought to be four major components of tumor stroma: 1) connective tissue matrix components; 2) vascular cells; 3) cells of the immune system; and 4) fibroblastic stromal cells (Hall et al., 2007). The latter have been designated as 'tumor associated fibroblasts' (TAF) (Kunz-Schughart and Knuechel, 2002a;Kunz-Schughart and Knuechel, 2002b), 'carcinoma associated fibroblasts' (CAF) (Orimo et al., 2005), or 'reactive stroma' (Rowley, 1998;Hall et al., 2007).

Bone marrow (BM) fibroblasts also play an important role in the development of stromal cell populations in tumors in mice (Direkze et al., 2004;Ishii et al., 2005). Fibroblast stromal cells secrete stromal derived factor 1 (SDF-1), which in turn promotes angiogenesis and tumor cell growth (Orimo et al., 2005). Tumor-associated stromal cells produce factors such as cytokines, growth factors, and matrix-degrading enzymes that biologically impact the tumor microenvironment (Silzle et al., 2004). Several other chemokines are known to be secreted by tumors, including vascular endothelial growth factor (VEGF), transforming growth factor (TGF) family members, fibroblast growth

factor (FGF) family members, platelet derived growth factor (PDGF) family members, monocyte chemotactic protein -1 (MCP-1), epidermal growth factor (EGF), and interleukin-8 (IL-8) (Nakamura et al., 2004).

Breast cancer cells have been reported to secrete the chemokines CXCR4, CCL5, and CCL2 (Muller et al., 2001;Kulbe et al., 2004). SDF-1 is expressed in many tumor cells like breast, lung, pancreatic, colon, prostate, neuroblastoma, glioblastoma, and ovarian carcinomas (Koshiba et al., 2000;Rempel et al., 2000;Geminder et al., 2001;Scotton et al., 2001;Muller et al., 2001;Schrader et al., 2002;Taichman et al., 2002;Burger et al., 2003;Hwang et al., 2003;Zeelenberg et al., 2003). While tumors secrete chemokine factors, they recruit stromal, vascular, bone marrow and other stem cells to the tumor; theoretically the recruited cells provide a scaffolding and source of nutrients (Tlsty and Hein, 2001;van Kempen et al., 2003;Kucerova et al., 2007).

Presence of chemokine receptors on various cells may aid in trafficking of these cells toward tumors. Low passage human bone marrow mesenchymal stem cells (MSC) have been shown to express the following chemokine receptors: CCR1, CCR7, CCR9, CXCR4, CXCR5, and CXCR6 (Honczarenko et al., 2006). MSCs cultured in serum-free medium express a number of chemokine ligands (CCL2, CCL4, CCL5, CCL20, IL-8, IL-12, CXCL8, CXCL12, and CX3CL1) (Honczarenko et al., 2006). Factors such as SDF-1 alpha, EGF, and PDGF been shown to enhance bone marrow MSC migration to tumor cells (Nakamizo et al., 2005). There is also abundant evidence that stem cells show tropism towards injured tissue or organ sites (Aboody et al., 2000;Natsu et al., 2004;Rojas et al., 2005;Lange et al., 2005;Phinney and Isakova, 2005;Sato et al., 2005;Silva et al., 2005), and can engraft and persist within tumor

microenvironments (Studeny et al., 2002;De et al., 2003;Studeny et al., 2004;Nakamizo et al., 2005;De et al., 2005;Rachakatla et al., 2007).

GENE THERAPY

During the last decade, gene therapy has become an emerging area of research in the medical and pharmaceutical field (Rawat et al., 2007). Gene therapy can be defined as transfer of the new genetic material into an individual's cells and tissues for therapeutic applications by altering function at cellular or molecular level (Goessler et al., 2006). The therapeutic gene needs a carrier vector to deliver it to the target cells. Each vector has unique properties and various gene carrying vectors have been investigated for efficient intracellular delivery. There are variety of vectors to deliver the genes to the target cells, such as viral vectors and non viral vectors. The most common viral vector systems are retroviruses (Kohn et al., 1989;Gilboa, 1990;Cournoyer and Caskey, 1993), adenoviruses (Rowe et al., 1953;Ballay et al., 1985;Yamada et al., 1985;Stewart et al., 1991;Lemarchand et al., 1992;Rosenfeld et al., 1992), adeno-associated viruses (AAV) (Blacklow et al., 1968;Cheung et al., 1980;Podsakoff et al., 1994), and herpes simplex viruses (Glorioso et al., 1995). The non viral methods include cationic liposomes (Thierry and Dritschilo, 1992;Bennett et al., 1992;Ropert et al., 1993), direct injection of naked DNA plasmids (Zhang et al., 2001), electroporation (Magin-Lachmann et al., 2004), and antisense oligonucleotides (Thierry and Dritschilo, 1992;Bennett et al., 1992).

The characteristics of both viral and non viral gene delivery systems are summarized in Table 1.1 (Romano et al., 1999; Goessler et al., 2006).

Vectors	Advantages	Disadvantages
Retrovirus	• Long term expression • Only infect dividing cells • No toxic effect on infected cells • Large DNA inserts possible	• Inflammatory/ immune response • Potential insertional mutagenesis • Relatively low transfection efficiency
Adenovirus	• Infect dividing as well as non dividing cells • Relatively long term expression • No toxic effect on infected cells • Large DNA inserts possible	• Inflammatory/ immune response • Lack of permanent expression • Complicated vector genome
Adeno-associated viruses	• Infect dividing as well as non dividing cells • Relatively long term expression • No toxic effect on infected cells	• Small DNA insert size • Inflammatory/ immune response • Lack of permanent expression • Complicated vector genome • Not well characterized
Herpes simplex virus	• Neurotropic • Large DNA inserts possible • Long term expression	• Neurotropism limits use • Relatively low transfection efficiency • Potential wild type breakthrough
Direct injection (naked DNA/ plasmids)	• Technically simple • Non toxic • Local delivery • Infect dividing as well as non dividing cells	• Unable to target specific cells • Relatively low transfection efficiency • Low long term transfection rates
Electroporation	• Nontoxic • Large DNA inserts possible • Infect dividing as well as non dividing cells	• Unable to target specific cells • Need for electric impulses • Relatively low transfection efficiency • Complex equipment
Liposom	• Technically simple • Large DNA inserts	• Unable to target specific cells

es	possible • Local delivery • Non immunogenic	• Relatively low transfection efficiency • Low long term transfection rates
Antisens e oligonucleotides	• Technically simple • Sequences can be ordered commercially • Nontoxic	• Very short term • Not always successful in decreasing expression • Non specific

Table 1.1 Summary of viral and non viral gene delivery systems

Though the viral gene delivery systems are associated with an increased risk of virus-associated toxicity (Salyapongse et al., 1999), they have been engineered for safety by making them replication incompetent (Robbins and Ghivizzani, 1998). Retroviruses are used for ex vivo gene therapy applications only as they are not efficient in infecting non dividing cells (Danos and Heard, 1992;Robbins and Ghivizzani, 1998). Adeno-associated virus, adenovirus, herpes simplex virus, as well as the non viral vectors are efficient in infecting both diving and non dividing cells and are used for either direct *in vivo* or ex vivo delivery (Oligino et al., 2000).

Non viral vectors are inexpensive and can be produced in large amounts. These vectors are safe and have a low immunogenicity (Oligino et al., 2000). However, the disadvantage of non viral vectors over viral vectors is that they have relatively low transfection efficiency (Salyapongse et al., 1999).

POTENTIAL ROLE OF STEM CELLS IN HOMING AND GENE THERAPY

Stem cells migrate toward wounds and other areas of pathology and have been shown to be effective gene-delivery vehicles for targeted cancer therapy (Aboody et al.,

2000;Studeny et al., 2002;Brown et al., 2003;Studeny et al., 2004;Nakamura et al., 2004;Natsu et al., 2004;Rojas et al., 2005). The first report describing stem cell tropism for tumors involved NSCs; Aboody and colleagues reported that these stem cells had the ability to migrate to experimental intracranial gliomas (Aboody et al., 2000). The authors have shown that NSCs implanted into intracranial gliomas established in mice, distributed themselves throughout the tumor mass, and infiltrated the tumor cells but were not seen in any other healthy brain tissue. NSCs transplanted intracranially at distant sites from the tumor also migrated toward the tumors (Aboody et al., 2000). Cellular homing and migration of NSCs to tumor cells is affected by the expression of CXCR4, a chemokine receptor (Gupta et al., 1998;Muller et al., 2001;Schrader et al., 2002;Lazarini et al., 2003).

Genetic modification of stem cells has facilitated their use as drug delivery vehicles for antitumor compounds. Several investigators have genetically modified NSCs with antitumor agents such as interleukin 12 (IL-12), interferon-γ, and tumor necrosis factor-related apoptosis inducing ligand (TRAIL). When transplanted into glioma rodent models these modified cells increased the survival rates of the animals (Ehtesham et al., 2002a;Ehtesham et al., 2002b;Ehtesham et al., 2002c;Shah et al., 2003;Yang et al., 2004;Shah et al., 2005). Ehtesham *et al.* have infected NSCs with either adenoviral vector expressing the gene for murine IL-12 (AdmIL-12) or beta-galactosidase (AdLacZ). To determine the homing ability, NSCs infected with AdLacZ (NSC-LacZ) were injected into the contra- lateral corpus striatum 7 days after establishing intracranial glioma. NSCs preferentially migrated towards the tumor mass, and did not migrate to adjacent normal tissue (Ehtesham et al., 2002b). These authors

have also investigated the therapeutic ability of NSCs infected with AdmIL-12 (NSC-IL-12). For tumor inoculation, GL26 glioma cells were injected into the right corpus striatum, and 2 days later, NSC-IL-12 were injected directly into the established tumor. They found that NSC-IL-12 delayed the tumor growth and prolonged the survival period of tumor-bearing animals. Nearly 30% of animals survived for more than 60 days after tumor implantation (Ehtesham et al., 2002b). A similar study by Yang *et al*. showed that NSCs expressing IL-12 have a strong antitumor effect (Yang et al., 2004). MRI studies showed that NSCs injected directly into the tumor gradually decreased the tumor in the tumor-bearing rats and the survival rate was significantly prolonged when compared to controls (Yang et al., 2004). NSCs engineered with a retrovirus expressing cytosine deaminase (CD) were transplanted into intracranial glioma established in nude mice; following systemic treatment with pro drug, 5-fluorocytosine (5-FC). Since CD metabolizes the relatively non toxic prodrug 5-FC to the highly toxic 5-fluorouracil (5-FU), tumor burden was drastically reduced (80%) as compared with untreated animals. *In vitro* co-culture experiments of CD bearing NSCs and glioma cells in combination with 5-FC significantly increased tumor cell death (Aboody et al., 2000). In more recent studies, NSCs were infected with retrovirus expressing CD (CD-NSCs) and co-cultured CD-NSCs with medulloblastoma cells. Following 5-FC treatment, the CD-NSCs inhibited the cancer cells significantly (Shimato et al., 2007). These results were consistent with an *in vivo* leptomeningeal dissemination model, where CD-NSCs injected directly into cerebrospinal fluid migrated and engrafted into the tumor area and showed an antitumor effect after systemic injection of 5-FC (Shimato et al., 2007).

Bone marrow MSCs have been shown to exhibit a tropism for damaged or rapidly growing tissues as well as tumors (Azizi et al., 1998;Kopen et al., 1999;Studeny et al., 2002;Studeny et al., 2004;Nakamizo et al., 2005). Bone marrow derived mesenchymal stem cell based gene therapies for cancer models have received considerable scrutiny in the last few years (Studeny et al., 2002;Lee et al., 2003;Zhang et al., 2004;Nakamura et al., 2004). The bone marrow cells that adhere have good proliferating capacity and have been shown to contribute to stroma formation even in sites that are remote from bone marrow (Hamada et al., 2005). The increased cell turnover triggered at the time of tissue damage or tumor growth may also help in the successful engraftment of MSC in tissues (Hall et al., 2007). *In vitro* chemotaxis assay studies revealed that MSCs migrate in response to expression of SDF-1 and fractalkine (CX3CL1), a membrane-bound glycoprotein (Ji et al., 2004). Magnetic resonance imaging (MRI) studies in experimental glioma rat models transplanted with neural progenitor cells and bone marrow MSCs either directly into the brain or intravenous injections showed extensive migration of MSCs towards tumor mass and infiltrated tumor cells (Zhang et al., 2004). Several viral vectors have been used to deliver transgenes into MSCs.

Nakamura *et al*. have reported that MSCs transduced with recombinant adenovirus expressing interleukin-2 (IL-2), when transplanted into glioma-bearing rats showed tropism to tumors, reduced the tumor burden, and prolonged the survival of the rats (Nakamura et al., 2004;Hamada et al., 2005). In similar experiments, MSCs transduced with adenovirus IL-2 and injected into tumor bearing mice have increased the immune response (CD8 mediated tumor specific immunity) and significantly delayed

tumor growth when transplanted into a B16 melanoma mouse model (Stagg et al., 2004).

An example of a protein successfully delivered via stem cells for an anti-tumor effect is interferon beta (IFN-β) (Studeny et al., 2002;Studeny et al., 2004). This cytokine has potent pro-apoptotic (Lokshin et al., 1995;Chawla-Sarkar et al., 2001) and antiproliferative (Wong et al., 1989;Johns et al., 1992) effects *in vitro*. However, IFN-β alone often cannot be used effectively as cancer therapy because of its excessive toxicity when administered systemically at high doses (Salmon et al., 1996;Einhorn and Grander, 1996;Buchwalder et al., 2000), and moreover it has a short half-life. Studeny *et al.* have shown that high toxicity can be reduced by transplanting MSCs infected with adenovirus expressing IFN-β (MSC-IFN-β) to tumors. A SCID mouse model bearing MDA 231 lung tumors was used to examine the effects of recombinant IFN-β on the growth of the tumors and their survival *in vivo*. Tumor bearing mice injected with MSC-IFN-β cells contributed to stroma formation and prolonged the survival of mice when compared to untreated mice. The survival was prolonged due to the suppression of tumor growth by MSC-IFN-β cells, through the local production of IFN-β in the tumor microenvironment. Mice injected with recombinant human IFN-β did not prolong the survival of the tumor bearing mice. The same report showed that co-cultures of MSC-IFN-β cells with MDA 231 breast carcinoma cells and A375SM melanoma cells inhibited the tumor cell growth when compared to growth of tumor cells cultured alone (Studeny et al., 2004). Thus stem cells can be used as a platform for targeted delivery of therapeutic proteins to the cancer sites.

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CHAPTER 2 - Comparison of transduction efficiency in human UCMS cells by wild type and fiber-modified adenoviruses

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Abstract

Adenovirus vectors have the ability to transduce both dividing and non dividing cells. However, most adenoviruses can transduce successfully only those cells that express Coxsackie-Adenovirus Receptor (CAR) and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Efficient adenoviral (Ad5) transduction depends on binding of the fiber protein of the virus to the CAR, and their subsequent internalization, mediated by viral penton base binding with target cell integrins. On the other hand, fiber-modified adenovirus can be effective in overcoming these limitations; CAR independent targeting strategies, such as fiber-modification with variable lengths of polylysine (K4, K7 and K21). In this study, we compared the transduction efficiency in human umbilical cord matrix stem (UCMS) cells by wild type and fiber-modified adenoviruses. UCMS cells are derived from Wharton's jelly found between the vessels of umbilical cord. To compare the transduction efficiency, UCMS cells were transduced with either wild type (Ad5) or fiber-modified adenovirus (Ad5/K4, Ad5/K7, Ad5/K21) beta galactocidase (β-Gal). All the fiber-modified adenoviruses transduced UCMS cells more efficiently when compared to wild type adenovirus. These results suggest that the fiber-modified viruses will help in designing safer gene therapy methods and can achieve higher clinical efficacy when compared with wild type adenoviruses.

Introduction

Recombinant adenoviral vectors are used most commonly for *in vitro* and *in viv*o gene therapeutic experiments in numerous gene transfer studies. Adenovirus vectors

have the remarkable ability of transducing not only the quiescent (or dormant) cells but also the rapidly proliferating cells (Kovesdi et al., 1997;Benihoud et al., 1999). However, most prevalently used adenovirus can transduce successfully only in those cells that express Coxsackie-Adenovirus Receptor (CAR) (Bergelson et al., 1997;Tomko et al., 1997;Bergelson et al., 1998) and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Wickham et al., 1993;Huang et al., 1996). The adenovirus infection of the target cells involves two unique steps. The first step is the binding of the C terminal knob domain of fiber protein of the virus to the CAR on the surface of the target cells (Bergelson et al., 1997;Tomko et al., 1997). Following that, the RGD (Arg-Gly-Asp) motif of the penton bases bind to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, expressed on most cell types. These interactions enable the internalization of the virus in the target cells via receptor mediated endocytosis (Wickham et al., 1993;Wickham et al., 1994). Since the presence of CAR is the first determinant of adenoviral infection, the interaction of the fiber knob with CAR on the cell is the important factor for the entry of the adenovirus into the cell.

However, the adenoviral vector mediated gene transfer is limited due to the absence or extremely low expression of CAR in certain kinds of cells, including differentiated airway epithelium, skeletal muscle cells, smooth muscle cells, peripheral blood cells, hematopoietic stem cells and most mouse derived cells (Mentel et al., 1997;Marini et al., 1999;Wickham, 2000;Rebel et al., 2000). If the cells are transduced with high multiplicities of infection (MOIs) or stimulated by numerous other growth factors, then the stem cell infection can be achieved with adenovirus serotype 5 (Ad5) (Mackenzie et al., 2000). However these may induce differentiation of the cells and loss

of the most pluripotent population (Mackenzie et al., 2000) and following infection there may be a high proportion of cell death (Mackenzie et al., 2000).

The limitation imposed by the expression of CAR in the target cells for adenovirus infection can be potentially overcome by modifying the adenovirus fiber protein. One of the many promising strategies to overcome this limitation, involves addition of foreign peptides to the H1 loop or C terminus of the fiber knob, such as mutant fiber proteins containing RGD peptides (Wickham et al., 1997;Dmitriev et al., 1998;Krasnykh et al., 1998;Hidaka et al., 1999;Koizumi et al., 2001;Mizuguchi et al., 2001) or polylysine repeats (K3, K7, K21) (Wickham et al., 1997;Hidaka et al., 1999;Bouri et al., 1999;Gonzalez et al., 1999b). Nevertheless, modification of Ad5 knob fiber tropism forms the basis for the transduction of normal and hemotopoietic cells (Yotnda et al., 2001;Yotnda et al., 2004), it is still unclear whether fiber-modified virus will prove optimal for umbilical cord matrix stem (UCMS) cells.

UCMS cells are derived from Wharton's jelly found between the vessels of umbilical cord and have been shown to have properties similar to bone marrow-derived mesenchymal stem cells (Weiss et al., 2006). They can be isolated in large numbers in a short time and thus potentially represent an abundant source of cells for therapeutic use.

Here, we have compared the transduction efficiency in human UCMS by wild type Ad5 and fiber-modified viruses. We determined which vectors transduced the highest percentage of cells with lowest viral particle to cell ratio and compared their levels of expression.

Material and methods

Cell culture and antibodies

Human umbilical cord matrix stem (UCMS) cells were harvested from term deliveries at the time of birth with the mother's consent. The methods to isolate and culture human UCMS cells were previously described (Weiss et al., 2006). Human UCMS cells were maintained in defined medium (DM) (Weiss et al., 2006).

Rabbit anti β-Gal polyclonal antibody was purchased from Becton Dickinson, NJ) and Alexa Fluor 488 conjugated secondary antibody was purchased from Molecular Probes, CA.

Adenoviral transduction of human UCMS cells with wild type and fiber-modified β-Gal adenoviruses

Human UCMS cells were transduced with either wild type or fiber-modified β-Gal recombinant adenovirus (Studeny et al., 2002). For transduction, UCMS cells were plated at 50,000 cells per well in a 12-well plate and twenty four hours later, the human UCMS cells were washed twice with DMEM without serum and cells were incubated with DMEM containing either wild type (Ad5) or fiber-modified (Ad5/K4, Ad5/K7, Ad5/K21) adenovirus β-Gal at various amounts (50, 100, 200, 400, 800, 1600, 3200, 6400, 12500, 25000 and 50000) of adenoviral particles (VP) per cell for 4 hours at 37°C. Medium containing 5% FBS was added after incubation. Production of β-Gal protein was determined 48 hours after transduction, using immunocytochemistry.

Immunocytochemistry

For immunofluorescence staining, β-Gal transduced UCMS cells were fixed by treating with buffered neutral formalin (BNF). This was followed by washing the fixed cells with three changes of phosphate buffered saline-0.2% Triton X-100 (PBS-TX). The cells were blocked with 5% normal goat serum in PBS-TX for 30 minutes, and later incubated with primary antibody, anti-rabbit β-Gal antibody (1:1000 Becton, Dickinson, NJ), in PBS-TX for 60 min. The cells were then washed three times with PBS-TX and incubated with Alexa Fluor 488 conjugated secondary antibody (1:1000, Molecular Probes, CA) for 45 minutes. The antigens were localized using epifluorescence microscopy (Nikon Eclipse) and images were captured using a Roper Cool Snap ES camera and Metamorph 7.

Results

Effect of transduction efficiency of wild type and fiber-modified adenovirus β-Gal in human UCMS cells

To investigate the efficiency of gene transfer of wild type and fiber-modified viruses in human UCMS cells, we transduced UCMS cells with either wild type or fiber-modified adenoviruses β-Gal. Forty eight hours after infection, the cells were analyzed by immunocytochemistry for β-Gal expression.

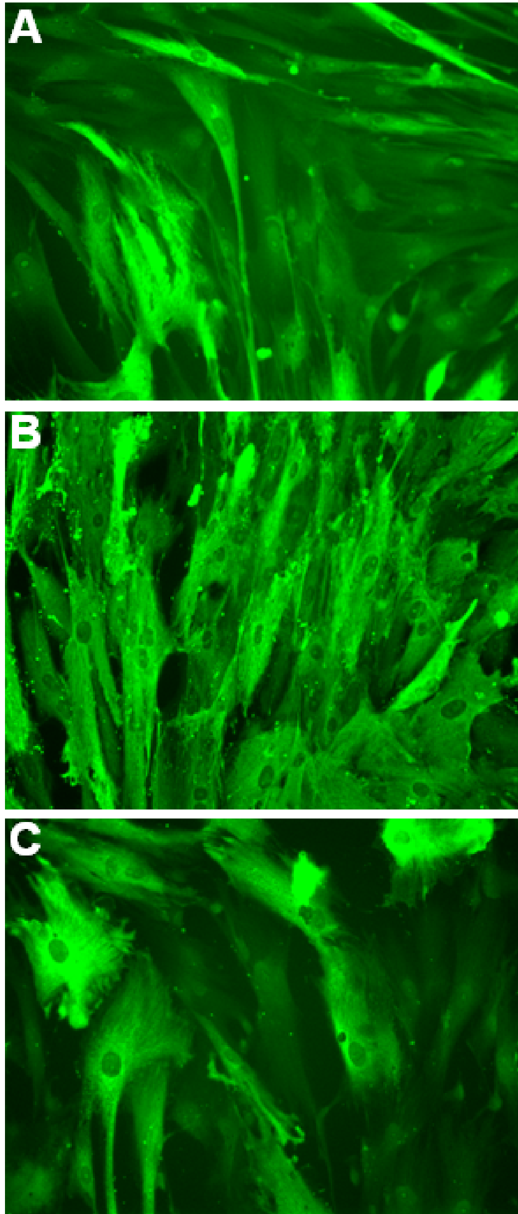

Figure 2.1 Adenoviral mediated expression of β-Gal.

Human UCMS cells transduced with fiber-modified β-Gal adenoviruses and immunostained with anti β-Gal antibody (green). A. Ad5/K4: B. Ad5/K7: C. Ad5/K21.

The expression β-Gal in UCMS cells was confirmed by immunocytochemistry (Figure 2.1). Ad5/K4 mediated gene transfer was the most efficient, with a maximum of

100% positive expression when transduced with 200 VP/cell (Figure 2.2). Ad5/K7 mediated gene transfer of UCMS cells was 100% when transduced with 800 VP/cell and Ad5/K21 mediated gene transfer was 100% at 6400 VP/cell transduction (Figures 2.3 and 2.4). Wild type adenovirus resulted in negligible transduction regardless of dose of VP/cell (no expression even at 50000 VP/cell). These results suggest that transduction efficiency of fiber-modified adenoviruses were very effective when compared with wild type adenoviruses in human UCMS cells.

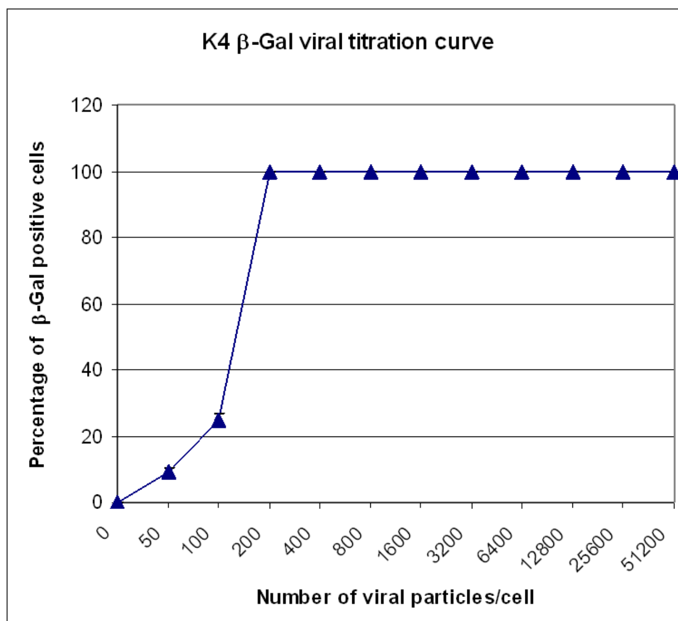

Figure 2.2 Human UCMS cells transduced with Ad5/K4.

The maximum expression was obtained with 200 VP per cell. The data are represented as mean ± standard deviation (SD) on graphs.

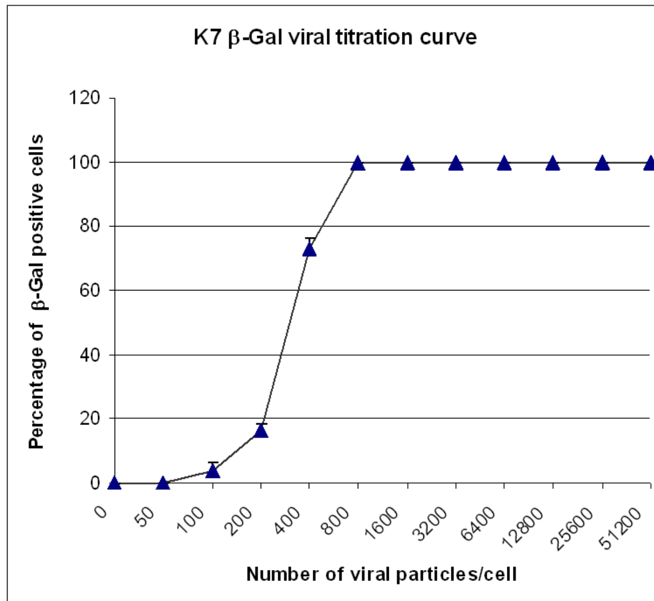

Figure 2.3 Human UCMS cells transduced with Ad5/K7.

The maximum expression was obtained with 800 VP per cell. The data are represented as mean ± SD on graphs.

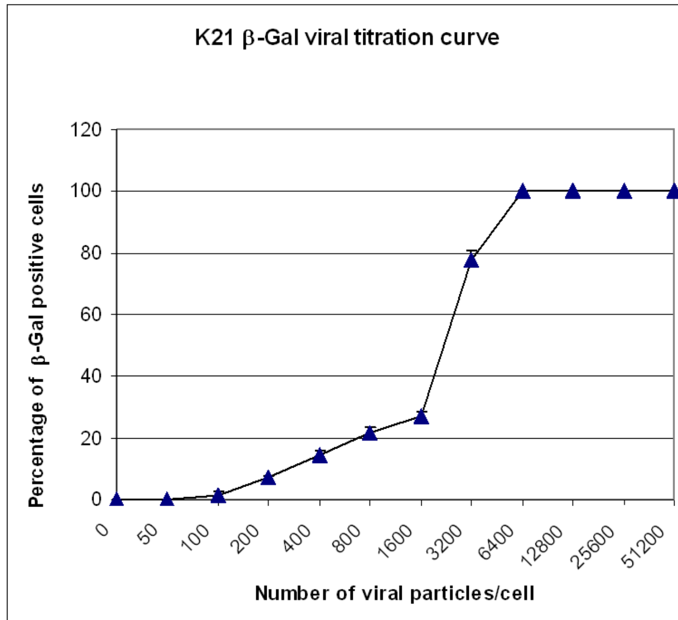

Figure 2.4 Human UCMS cells transduced with Ad5/K21.

The maximum expression was obtained with 6400 VP per cell. The data were represented as mean ± SD on graphs.

Discussion

The characteristic of Ad5 to transduce either quiescent or proliferating cells and to produce a high level of transient gene expression makes it an indispensable vector for gene therapeutic protocols. However this virus works inadequately in many types of cells because they lack necessary receptor molecules for binding (CAR) (Bergelson et al., 1997) and for internalization (integrins) (Wickham et al., 1993).

There have been other strategies to infect normal and malignant cells of the hematopoietic lineage including the usage of lipofectamine (Byk et al., 1998), bi specific antibodies that target both adenovirus epitopes and cell antigens, biotinylated adenoviruses (Smith et al., 1999), or adenoviruses with heparin sulfate binding domains (Gonzalez et al., 1999a). In all these studies, it has been generally observed that large amounts of vector were needed and that there has not been much proven success with highly primitive stem cell populations (Smith et al., 1999;Gonzalez et al., 1999a). Several studies have shown a way to overcome these restrictions with the use of fiber-modified adenovectors (Yotnda et al., 2001;Yotnda et al., 2004). The limitations of the other alternatives available for this application made us concentrate on adenovectors.

Retroviruses have shown to be integrating vectors, and may intensify the cellular dysfunction in already stable cells (Li et al., 2002). Lentiviral vectors were used in the transfection of quiescent human acute lymphoblastic leukemia and acute myeloid leukemia cells, but the productivity is minimal and may require co-culturing with stroma and/or cytokines (Biagi et al., 2001) whereas the adenovectors have the abilities to transduce non dividing cells, are non integrating, and provide high levels of transgene expression.

Since this study was done to compare the efficiency of transduction, we standardized infection in terms of number of viral particles per cell, duration, and temperature of transduction (4 hr 37°C). Of the vectors tested, the modified Ad/K4 consistently transduced up to 100% of UCMS cells at a low viral particle to cell ratio and thus constitutes the most efficient of the vectors that were under study.

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CHAPTER 3 - *In vitro* migration of human umbilical cord matrix stem cell in response to chemotactic signals from cancer cells

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Abstract

Umbilical cord matrix stem (UCMS) cells are derived from mesenchyme-like cushioning material called 'Wharton's jelly' found between the vessels of the umbilical cord. The umbilical cord matrix contains an inexhaustible, non-controversial source of stem cells. Chemokines control immune and inflammatory cellular migration and play an important role in tumor progression. In this study, we examined the role of MDA 231 human breast carcinoma cells and some chemokines in trafficking of human UCMS cells in an *in vitro* model of cell migration. To investigate the migratory nature of human UCMS cells towards MDA 231 cells, UCMS cells were cultured with or without MDA 231 cells for 24 hours. UCMS cells were found to migrate in a dose dependent manner with an increasing number of MDA 231 cells. Next, we evaluated the effect of two candidate chemokines, SDF-1 and VEGF, on human UCMS cells by challenging them with increasing doses of the two factors. Both SDF-1 and VEGF increased the migration of UCMS cells in a dose dependent manner. Because the UCMS cells respond positively to SDF-1 and VEGF , these factors may be involved in the *in vivo* trafficking of the stem cells to MDA 231 metastatic lung tumors. Within the tumor, cancer cells or cancer-associated stromal cells might be releasing chemokine factors such as SDF-1 and VEGF which promote UCMS cell migration towards the tumor cells *in vitro*. Since the UCMS cells respond to chemotactic signals from tumors, they can be used as gene delivery vehicles in targeting the tumors *in vivo*.

Introduction

The umbilical cord contains an inexhaustible, non-controversial source of stem cells. Worldwide, millions of umbilical cords, each containing millions of stem cells, are

routinely discarded after birth. Multipotent stem cells called umbilical cord matrix stem (UCMS) cells are isolated from the mesenchyme-like cushioning material ‘Wharton’s jelly’ found between the vessels of the umbilical cord (Mitchell et al., 2003). These cells resemble stem cells from several other sources but are also unique in some properties. Several characteristics argue for their potential use in cell-based therapeutic solutions for human or animal diseases: Large numbers of cells that can be isolated from a single umbilical cord, the noninvasive postnatal (hence, non-controversial) harvest of cells, and the inexhaustible supply of umbilical cords (Weiss et al., 2006;Karahuseyinoglu et al., 2007). Thus, they may offer an immediate avenue for cytotherapy, when time is of the essence for such therapy, for example in cases of malignant neoplasia.

Tumors are sites of inflammatory cytokine and chemokine production (Hall et al., 2007). Chemokines are low molecular weight proteins secreted by cells and characteristically have four conserved cysteine residues. They are pro-inflammatory, play an important role in leukocyte maturation, trafficking, angiogenesis, migration of cells during tissue development, and in homing of T and B lymphocytes (Taub and Oppenheim, 1994;Bokoch, 1995;Premack and Schall, 1996;Luster, 1998). Chemokines are classified into four sub families based on spacing of their first two cysteine residues; α-chemokines (CXC), β-chemokines (CC), γ-chemokines (C) and δ–chemokines (CXXXC) (Taub and Oppenheim, 1994;Bokoch, 1995;Premack and Schall, 1996;Luster, 1998). Stromal cell-derived factor 1a (SDF-1) is a member of the α-chemokine subfamily and its cognate receptor is CXCR4 (Hamada et al., 1996;Feng et al., 1996;Smith et al., 2004). CXCR4 expression was observed to be upregulated in glioblastomas and breast cancer cells (Sehgal et al., 1998;Muller et al., 2001;Kulbe et

al., 2004). SDF-1 plays an important role in homing as shown by studies on engraftment of hematopoietic stem cells to bone marrow (Peled et al., 1999) and engraftment of metastatic breast and prostate cancer cells to bone and bone marrow (Muller et al., 2001). Vascular endothelial growth factor (VEGF) which belongs to the PDGF family, is a signaling protein involved in both vasculogenesis and angiogenesis (Ferrara and Gerber, 2001). Active angiogenesis is a major hallmark of tumors (Harrigan, 2003). Schmidt *et al* have shown that VEGF is responsible for migration of neural stem cells (NSCs) to distant sites in brain tumors (Schmidt et al., 2005).

Tumor tropism is known to be mediated by cytokines, including VEGF, TGF family members, FGF family members, PDGF family members, MCP-1, EGF, and interleukin-8 (Nakamura et al., 2004). Several investigators have shown that tumors, by secreting chemokine factors, recruit stromal, vascular, bone marrow and other stem cells to the tumor; theoretically the recruited cells provide a scaffolding and source of nutrients (Tlsty and Hein, 2001;van Kempen et al., 2003;Kucerova et al., 2007). There has been abundant evidence that mesenchymal stem cells (MSC) show tropism towards injured tissue or organ sites (Natsu et al., 2004;Rojas et al., 2005;Lange et al., 2005;Phinney and Isakova, 2005;Sato et al., 2005;Silva et al., 2005), and can engraft and persist within tumor microenvironments (Studeny et al., 2002;De et al., 2003;Studeny et al., 2004;Nakamizo et al., 2005;De et al., 2005;Rachakatla et al., 2007). The chemokines, such as SDF-1 alpha, EGF, and PDGF were shown to enhance bone marrow MSC migration to tumor cells (Nakamizo et al., 2005).

In vitro chemotaxis assay studies revealed that MSCs migrate in response to expression of SDF-1 and fractalkine (CX3CL1), a membrane-bound glycoprotein (Ji et

al., 2004). Several other studies have shown that cellular homing and migration of NSCs to tumor cells is affected by the expression of CXCR4, a chemokine receptor (Gupta et al., 1998;Muller et al., 2001;Schrader et al., 2002;Lazarini et al., 2003). Cell invasion assays using adipose tissue-derived mesenchymal stem cells (AT-MSC) have shown that these cells also have tropism towards tumor cells (Kucerova et al., 2007). Schmidt *et al* verified *in vitro* that tumor cells upregulated VEGF and recombinant VEGF stimulated NSC migration in a dose dependent manner (Schmidt et al., 2005).

In the present study, we investigated migratory ability of human UCMS cells towards MDA 231 human breast carcinoma cells. Next, we examined the effect of chemokines, SDF-1 and VEGF, on the migration of human UCMS cells. Here, we show that human UCMS cells migrate towards MDA 231 cells in a dose dependent manner and there was a significant stimulatory effect of recombinant SDF-1 and VEGF on UCMS cell migration.

Methods

Cells and chemokines

Human umbilical cord matrix stem (UCMS) cells were harvested from term deliveries with the mother's consent. The methods to isolate and culture human UCMS cells were previously described (Weiss et al., 2006). Human UCMS cells were maintained in low serum medium (LSM), a mixture of 56% low glucose Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen), 37% MCBD 201 (Sigma; St. Louis, MO) and 2% fetal bovine serum (FBS, Atlanta Biologicals Inc, Georgia) containing 1x insulin-transferrin-selenium-X (ITS-X, Invitrogen, CA), 1x ALBUMax1 (Invitrogen, CA), 1x Pen

/Strep (Invitrogen, CA), 10nM dexamethasone (Sigma, MO), 100μM ascorbic acid 2-phosphate (Sigma, MO), 10ng/ml epidermal growth factor (EGF, R&D systems, Minneapolis), and 10ng/ml platelet derived growth factor-BB (PDGF-BB, R&D systems, MN), at 37°C in a humidified atmosphere containing 5% carbon dioxide. Human UCMS cells were labeled with the fluorescent dye SP-DiI (Molecular Probes, CA) to a final concentration of 10μg/ml of culture medium (Rachakatla et al., 2007), prior to the cell migration assay. MDA 231 human breast carcinoma cells were obtained from M.D. Anderson Cancer Center (Houston, TX) (Studeny et al., 2002). They were maintained in DMEM (Invitrogen, CA), 1x Pen/Strep (Invitrogen, CA), and 10% FBS (Atlanta Biologicals Inc, GA), at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Recombinant murine stromal cell-derived factor-1 (SDF-1) and murine vascular endothelial growth factor (VEGF) were purchased from Peprotech Inc. (Rock Hill, NJ).

Transduction of human UCMS cells with adenovirus expressing human interferon beta (IFN-β)

Successful *in vivo* gene therapy using stem cells depends on delivery of therapeutic agents to suitable targeted cells; for this reason we investigated whether transgene expression (IFN-β) might have an effect on the migration and homing ability of UCMS cells. UCMS cells were transduced using fiber-modified IFN-β recombinant adenovirus (Studeny et al., 2002). For transduction, the human UCMS cells were washed twice with DMEM without serum and cells were incubated with DMEM containing adenovirus at 100,000 plaque forming units (PFU) per cell for 4 hours at 37°C. Medium containing 5% FBS was added after incubation and the UCMS-IFN-β cells were used for *in vitro* experiments the next day.

In vitro transwell cell migration assay

The cell migration assay was performed using 24-well double chamber culture plates, Transwell (Corning, NY). MDA 231 cells at various concentrations (50,000, 100,000 and 500,000 cells) were loaded in the lower chambers of the 24-well culture plates. 24 hours following the initial plating of MDA 231 cells, the media was changed and replaced with serum free medium (DMEM). Later, SP-DiI labeled human UCMS cells (1x 10^5 cells) cultured overnight were washed three times with DMEM and resuspended in DMEM and plated on the 8μm pore size inserts of the upper chamber and incubated for 24 hours at 37^0C in a humidified atmosphere containing 5% carbon dioxide. Each condition was run in duplicate and was repeated at least thrice. Results were evaluated by directly counting the number of migrated cells in each lower chamber using epifluorescence microscopy (Nikon Eclipse). Similar experiments were carried out using IFN-β engineered human UCMS cells. All data are presented as mean ± standard error on graphs.

To assess the individual effects of various chemokines, such as SDF-1 and VEGF in cell migration, experiments were carried out using increasing doses of chemokines (10, 100, 250, and 500ng/ml) in DMEM. The transwell membranes were coated with 25μg/ml of matrigel (Collabarative Biomedical products, MA) (Son et al., 2006). Media with various concentrations of chemokines was placed in lower chambers of the 24-well culture plates. Human UCMS cells (1x 10^5 cells) were suspended in DMEM with or without chemokines (SDF-1 or VEGF) and were plated on to the upper chambers and incubated for 24 hours. After incubation, the cells were scraped from upper surface of the membranes. The membrane was then fixed and stained using

hematoxylin. Once dry, the cells that migrated to the lower surface of the membrane were evaluated by directly counting from each transwell insert. Experiments were repeated thrice with similar results.

Statistical analysis

The means of the experimental groups were evaluated to confirm that they met the normality assumption. To evaluate the significance of overall differences in all *in vitro* groups, statistical analysis was performed with the student's t-test. A p-value less than 0.05 was considered as significant. All the data were represented as mean ± standard error on graphs. Statistical analyses were performed by Stat View software, version 5.0.1. (Cary, NC).

Results

In vitro migratory potential of human UCMS cells

The ability of human UCMS cells to migrate toward MDA 231 cells was evaluated by an *in vitro* cell migration assay model, where the SP-DiI loaded UCMS cells and MDA 231 cells are placed in the upper and lower chambers respectively in a double chamber Transwell culture plate and incubated for 24 hours. Lower chambers containing either DMEM with 10% FBS or DMEM with no MDA 231 cells served as positive and negative controls. Directional migration of human UCMS cells was significantly stimulated by either MDA 231 cells or DMEM with 10% FBS when compared with controls (DMEM and no MDA 231 cells)(Figure 3.1). Notably, migratory ability of UCMS cells increased in a dose dependent manner with increasing number of MDA 231 cells.

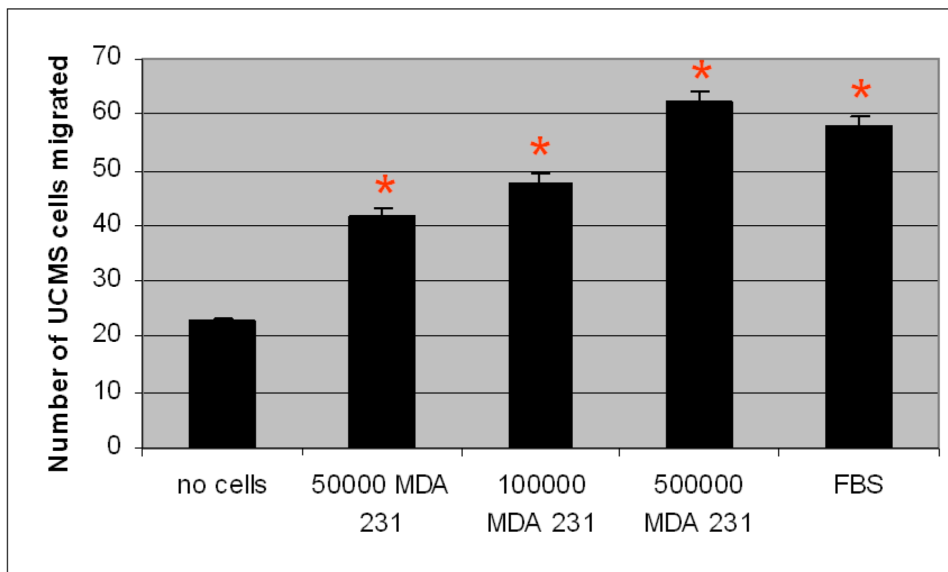

Figure 3.1 *In vitro* migration effect of UCMS cells toward MDA 231 human breast carcinoma cells.

UCMS cell migration increased in a dose dependent manner with increasing number of MDA 231 cells in the lower chamber. * Statistically significant (p-value less than 0.05).

Migration of human UCMS cells was not affected by transgene (IFN-β expression)

To investigate the migratory nature of UCMS-IFN-β cells towards MDA 231 cells, similar experiments were performed as mentioned above and the cells were replaced with UCMS-IFN-β cells and incubated for 24 hours. The number of cells migrated to the lower chamber increased in a dose dependent manner with increasing numbers of MDA 231 cells and there was significant difference ($P<0.05$) when compared with controls

(data not shown). Thus, engineering with IFN-β did not impair the migration capability ability of UCMS cells.

Effect of chemokines on migration of human UCMS cells

We hypothesized that the migration of human UCMS cells towards MDA 231 cells was due to several chemokines released by the MDA 231 cells. Therefore we evaluated the effect of the chemokine SDF-1, and VEGF on UCMS cell migration. The chemotactic effect of VEGF was increased in dose dependent manner and there was significant increase in UCMS cell migration when VEGF was added to DMEM in the lower chamber when compared to control (DMEM with out VEGF) (Figure 3.2). Similar chemotactic effect was observed when SDF-1 was added to DMEM in lower chamber. There was significant increase in UCMS cell migration when SDF-1 was added when compared with control (DMEM without SDF-1) (Figure 3.2). We also looked at the migratory ability of UCMS cells, when either SDF-1 or VEGF was added in both upper and lower chambers of the transwell plates and we observed that the UCMS cell migration was not significant enough when compared to control (DMEM alone). These results showed that VEGF and SDF-1 were significant and there may be several other chemokines along with these chemokines which may be responsible for the migratory effect of UCMS cells towards MDA 231 cells.

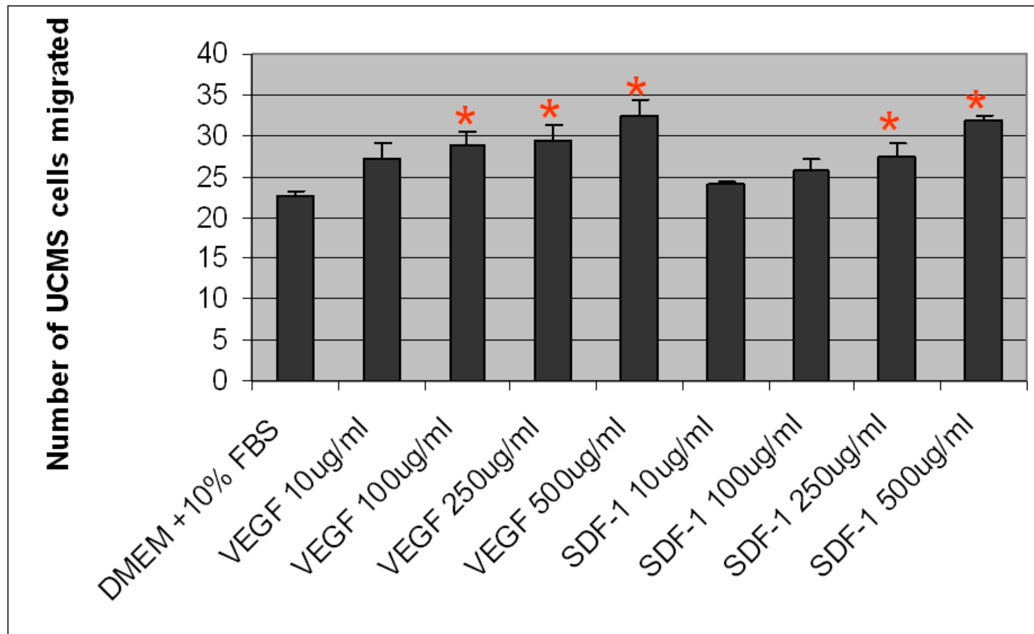

Figure 3.2 *In vitro* migration effect of UCMS cells toward chemokines (SDF-1 and VEGF).

UCMS cell migration increased in a dose dependent manner with dose of chemokine in the lower chamber. * Statistically significant (p-value less than 0.05).

Discussion

The understanding of the mechanism of chemokines that regulates migration of human UCMS cells is crucial for future clinical studies. Here, we address the migratory ability of human UCMS cells in response to chemokines and MDA 231 cells. In our *in vitro* study, we showed that MDA 231 cells were able to attract human UCMS cells in a dose dependent manner, and this activation and migration of UCMS cells may be mediated by chemokines released into the media by MDA 231 cells. Moreover, *in vitro* studies showed that UCMS cells migrated and responded in a dose dependant manner SDF-1 and VEGF.

Human UCMS cells have been characterized by gene array and cytokine array and they were found to express chemokine receptors including SDFR1, TGFBR3, FGFR2 (Weiss et al., 2006). The finding that human UCMS cells express these receptors is in agreement with the migration of human UCMS cells to these chemokines. Although the SDF-1-CXCR4 system contributed to UCMS cell migration, it is unclear whether those receptors operate synergistically or if they act in response to distinct stimuli. Several other studies have reported that homing and differentiation of neural precursors in the adult brain are associated with active angiogenesis, where in VEGF acts as a chemo attractant (Louissaint, Jr. et al., 2002;Monje et al., 2002;Zhang et al., 2003). To our knowledge this is the first report showing *in vitro* chemotactic migration of human UCMS cells.

In our earlier studies, human UCMS cells have been shown to migrate *in vivo* to tumors in response to chemotactic signals released by the tumors (Rachakatla et al., 2007). There may be more than one migratory stimulus for human UCMS cells, therefore UCMS cells can be mobilized to tumor tissue naturally or induced to migrate to the tumor tissue. The possible candidates other than SDF-1 and VEGF may be cytokines such as transforming growth factor (TGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), monocyte chemoattractat protein-1, and IL-8 released from tumor or inflammatory tissue (Hellstrom et al., 1999;Tille and Pepper, 2002;Wang et al., 2002a;Wang et al., 2002b;Yu et al., 2003). These factors released from cancer cells and/or associated stromal cells have been shown to promote migration of endothelial cell and stromal cell progenitors from the bone marrow towards the cancer tissue (Coussens and Werb, 2002;De et al., 2003), enhancing the formation

of tumor stroma (Weaver et al., 1996). Similar mechanisms would be anticipated for migration of human UCMS cells *in vitro* towards tumor cells.

The data presented here provide conclusive evidence that human UCMS cells can migrate to specific chemokine gradients of SDF-1, VEGF and to MDA 231 released chemokines. Strategies to increase the functional expression of the chemokine receptors may be required to improve the further homing ability of UCMS cells to tumors.

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CHAPTER 4 - Development of human umbilical cord matrix stem cell-based gene therapy for experimental lung tumors

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Abstract

Umbilical cord matrix stem (UCMS) cells are unique stem cells derived from Wharton's jelly that have been shown to express genes characteristic of primitive stem cells. To test the safety of these cells, human UCMS cells were injected both intravenously and subcutaneously in large numbers into SCID mice and multiple tissues were examined for evidence of tumor formation. UCMS cells did not form gross or histological teratomas up to 50 days after post transplantation. Next, to evaluate whether UCMS cells could selectively engraft in xenotransplanted tumors, MDA 231 cells were intravenously transplanted into SCID mice, followed by intravenous transplantation of UCMS cells one and two weeks later. UCMS cells were found near or within lung tumors but not in other tissues. Finally, UCMS cells were engineered to express human interferon beta–designated "UCMS–IFN-β". UCMS–IFN-β cells were intravenously transplanted at multiple intervals into SCID mice bearing MDA 231 tumors and their effect on tumors was examined. UCMS–IFN-β cells significantly reduced MDA 231 tumor burden in SCID mouse lungs indicated by wet weight. These results clearly indicate safety and usability of UCMS cells in cancer gene therapy. Thus, UCMS cells can potentially be used for targeted delivery of cancer therapeutics.

Abbreviations:

UCMS cells, umbilical cord matrix stem cells; UCMS–IFN-β cells, interferon beta-expressing UCMS cells; ESCs, embryonic stem cells; IFN-β, interferon beta; SCID, severe combined immunodeficiency; DMEM, Dulbecco's modified eagle's medium; FBS, fetal bovine serum; DMSO, dimethylsulphoxide; PBS, phosphate buffered saline;

SP-DiI, sulfonated derivatives of Dialkyl indol dye; HEPA, high efficiency particulate air; EDTA, ethylenediaminetetraacetic acid; IACUC, institutional animal care and use committees; IBC, institutional biosafety committee.

Introduction

The umbilical cord contains an inexhaustible, non-controversial source of stem cells. Worldwide, millions of umbilical cords, each containing millions of stem cells, are routinely discarded after birth. Multipotent stem cells called umbilical cord matrix stem (UCMS) cells are isolated from the mesenchyme-like cushioning material called 'Wharton's jelly' found between the vessels of the umbilical cord (Mitchell et al., 2003). These cells resemble stem cells from several other sources but are also unique in some properties. The properties that promote their potential utility are the large number of cells recoverable from the umbilical cord, the noninvasive harvest following birth, and the abundant supply of umbilical cords (Weiss et al., 2006;Karahuseyinoglu et al., 2007). Thus, they may offer an immediate avenue for cytotherapy, when time is of the essence for such therapy, for example in cases of malignant neoplasia. UCMS cells have been transplanted into rodent neurodegenerative models (Weiss et al., 2003;Medicetty et al., 2004;Fu et al., 2006), have been extensively characterized (Weiss et al., 2006) and they express many of the genes expressed by primitive stem cells such as embryonic stem cells (ESCs) (Carlin et al., 2006). They can be differentiated into a variety of cell types including muscle (Conconi et al., 2006), cartilage (Wang et al., 2004), bone (Wang et al., 2004), neural (Mitchell et al., 2003) cells and have been used for tissue engineered artificial blood vessels and heart valves (Hoerstrup et al., 2002).

Previously, several types of stem cells have been shown to migrate selectively into tumors (Aboody et al., 2000;Studeny et al., 2002). Moreover, they have been engineered to secrete anti-tumor proteins, such as interferon beta (IFN-β), into the tumor microenvironment with favorable therapeutic effects in rodent animal models (Aboody et al., 2000;Studeny et al., 2002;Brown et al., 2003;Studeny et al., 2004;Nakamizo et al., 2005). In these cases, millions of stem cells have been transplanted systemically. However, similar approaches in humans will likely require an order of magnitude more cells to achieve a comparable effect. Since large numbers of UCMS cells can be obtained in a short time, they potentially could be a solution to this dilemma.

An example of a protein successfully delivered via stem cells for an anti-tumor effect is interferon beta (IFN-β)(Studeny et al., 2002;Studeny et al., 2004). This cytokine has potent pro-apoptotic (Lokshin et al., 1995;Chawla-Sarkar et al., 2001) and antiproliferative (Wong et al., 1989;Johns et al., 1992) effects *in vitro*. Although gene therapy using bone marrow- or neural stem cells has been successful in treating cancer mouse models, the preparation of these stem cells usually requires complex methods and a relatively long time.

In the present study, we investigated a key safety issue by transplanting large numbers of UCMS cells into SCID mice to assess possible teratoma or other tumor formation. Next, we tested UCMS cells for their ability to selectively engraft in lung tumors of SCID mice. Finally, we investigated whether UCMS cells, engineered to secrete IFN-β, could reduce lung tumor burden in SCID mice. We show here that UCMS cells themselves do not form tumors; they selectively engraft in lung tumors. In

addition, tumor burden in SCID mice bearing lung tumors is significantly reduced following systemic administration of human IFN-β-expressing UCMS cells.

Materials and methods

Tissue culture of human umbilical cord matrix stem cells and MDA 231 cells

Human umbilical cord matrix stem (UCMS) cells were harvested following deliveries at the time of birth with the mother's consent. The methods to isolate and culture human UCMS cells were previously described (Weiss et al., 2006).

MDA 231, a human breast carcinoma cell line that engrafts in the lung of nude mice (Fraker et al., 1984) was maintained in DMEM (Invitrogen, Carlsbad, CA), and 10% FBS (Mediatech, Herndon, VA) at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Adenoviral vectors and transduction of human UCMS cells

Since UCMS cells do not express the adenoviral receptor, fiber-modified viruses were used to allow efficient transduction (Studeny et al., 2002;Studeny et al., 2004). Recombinant adenoviral particles carrying human IFN-β was used (Studeny et al., 2002;Studeny et al., 2004). For transduction, the human UCMS cells were plated in T-75 flasks and infected when the cells reached 80-90% confluency. The cells were washed twice with DMEM without serum and incubated with DMEM containing adenovirus at 12,500 viral particles per cell (for adenovirus-IFN-β) for 4 hours at 37oC. The cells were cultured in UCMS media (a mixture of 56% low glucose DMEM (Invitrogen), 37% MCBD 201 (Sigma; St. Louis, MO) and 2% fetal bovine serum (FBS,

Mediatech, MTT 35-010-CV) containing 1x insulin-transferrin-selinium-A (ITS, Invitrogen), 1x ALBUMax1 (Invitrogen), 1x Pen /Strep (Invitrogen), 1x Amphotericin-B (Invitrogen), 10nM dexamethasone (Sigma), 50µM ascorbic acid 2-phosphate (Sigma), 10ng/ml epidermal growth factor (EGF, R&D systems, Minneapolis, MN), and 10ng/ml platelet derived growth factor-BB (PDGF-BB, R&D systems)) for 24 hours and then used for transplantation studies.

IFN-β ELISA

The amount of IFN-β secreted by UCMS–IFN-β cells into the media was quantified by a human interferon beta ELISA kit (PBL biomedical laboratories, New Jersey). UCMS cells were plated at 50,000 cells per well in a 12-well plate. UCMS cells were transduced with adenovirus IFN-β at various amounts (12,500, 6,400 and 3,200) of adenoviral particles per cell as described above. One day later, the IFN-β level in the medium was determined according to manufacturer's protocols using recombinant IFN-β as a standard.

In vitro effect of conditioned media from IFN-β expressing UCMS cells on MDA 231 cells

To condition the media, fresh media was added to subconfluent (15,000 cells/cm^2) UCMS cells or UCMS-IFN-β cells and incubated for 24 hrs prior to being removed, centrifuged and its bioactivity was examined. MDA 231 cells were plated in 6-well plates at a density of 160,000 cells per well with MDA 231 growth media (DMEM and 10% FBS). Twenty four hours later, the media was removed from all the wells and the MDA 231 media was replaced with either conditioned media from UCMS cells

alone, from UCMS-IFN-β cells or unconditioned UCMS media as a control. After 3 days, the cells were trypsinized and counted on a hemocytometer. The percentage of dead cells was estimated using trypan blue exclusion. Results were also expressed as the percentage of cell growth, calculated with the following formula (Studeny et al., 2004): (Number of MDA 231 cells co-cultured with conditioned media on day 3 – the number of MDA 231 cells on day 0)/(number of MDA 231 cells cultured in UCMS media on day 3 – number of MDA 231 cells on day 0) X 100. Three different isolates of UCMS cells were used in separate trials and each experiment was done in triplicate.

Immunohistochemistry staining

For immunofluorescence staining, tissue sections were washed with phosphate buffered saline-0.2% Triton X-100 (PBS TX) and fixed with 70% ethanol and acetone (1:1) . This was followed by washing with three changes of PBS TX. Tissue sections were blocked with 5% normal goat serum in PBS TX for 30 minutes, and followed by incubation with primary antibody, anti-human mitochondrial antibody (1:1000, Chemicon, Temecula, CA), in PBS TX for overnight. The tissues were then washed three times with PBS TX and incubated with Alexa Fluor 488 conjugated secondary antibody (1:1000, Molecular Probes, Carlsbad, CA) for 3 hours. The tissues were incubated for 30 min in Hoechst 33342 (1:100, Sigma) as a counter stain to label the nuclei followed by a triple rinse with PBS TX. The antigens were localized using epifluorescence microscopy (Nikon Eclipse) and images were captured using a Roper Cool Snap ES camera and Metamorph 7.

Fluorescent labeling of human UCMS cells

For transplanted human UCMS cell identification, the red fluorescent dye SP-DiI (Molecular Probes) was dissolved in dimethylsulphoxide (DMSO) at a concentration of 5mg/ml. SP-DiI dye was added to culture medium to a final concentration of 10 μg/ ml and human UCMS cells were labeled by adding 10 ml of medium with SP-DiI in a T-75 flask for 24 hours. Then, cells were washed with PBS, incubated with dye-free medium for 4 hours, and used for transplantations.

Evaluation of tumor formation by human UCMS cells

For the following three *in vivo* studies 5 week old female CB-17 SCID mice were obtained from Harlan laboratories (Indianapolis, IN). Mice were held for 1 week after arrival to allow them to acclimatize. They were housed in a barrier room and individual cages containing HEPA filters. Both subcutaneous and systemic transplantation of UCMS cells suspended in 200μl PBS were carried out under sterile conditions in a laminar flow hood in the barrier room. Animals were randomly assigned to experimental groups. The first set of mice were transplanted with 2×10^6 human UCMS cells (n=5) and 1×10^7 human UCMS cells (n=4) subcutaneously at the flank region. The remaining mice were transplanted with 2×10^6 human UCMS cells (n=5), 3×10^6 human UCMS cells (n=2), and 6.5×10^6 human UCMS cells (n=1) intravenously via the lateral tail vein. All the mice were sacrificed 50 days after transplantation by cervical dislocation. Lung, kidney, and liver were collected, snap frozen in liquid nitrogen chilled isopentane, and used for immunohistochemistry studies. All *in vivo* experiments were carried out with proper IACUC and IBC institutional approval.

Evaluation of selective engraftment of human UCMS cells

MDA 231 cells and UCMS cells were transplanted without anesthesia into the lateral tail vein using sterile conditions in a laminar flow hood in the barrier room. Animals were randomly assigned to experimental groups. For tumor induction, one set of mice were transplanted with 1 X 10^6 MDA 231 cells (n=8). This was followed by two injections of 1 X 10^6 fluorescently labeled UCMS cells on day 17 and day 24 after injection of MDA 231 cells. A second set of mice were transplanted with 2 X 10^6 MDA 231 cells (n=8). This was followed by two injections of 1 X 10^6 fluorescently labeled UCMS cells on day 11 and day 18. All the mice were sacrificed 8 days after the last UCMS cell transplant. Other groups included mice injected with fluorescently labeled UCMS cells alone (n=4), and mice injected with sterile PBS only (n=4) as controls. Lung weights of control and tumor-bearing animals were measured to estimate tumor burden. Lungs and other organs including spleen, liver, kidney, and bone marrow were also harvested as described above for immunohistochemical analysis.

Evaluation of the effect of IFN-β expressing human UCMS cells on the growth of MDA 231 xenografts

MDA 231 cells and UCMS cells were transplanted without anesthesia as described above. Animals were randomly assigned to experimental groups. For tumor induction, 2 x 10^6 MDA 231 cells were given via the lateral tail vein (n= 22). Fluorescently labeled UCMS–IFN-β cells (0.5 x 10^6, n=9) or fluorescently labeled UCMS cells (0.5 x 10^6, n=5) were transplanted on day 8 after tumor inoculation. The UCMS cells transplant was repeated twice at 1 week intervals subsequent to the first transplant. Experimental groups also included a negative control group that received

neither MDA 231 cells nor UCMS cells (n=6; PBS injections only), and a group with 3 weekly injections of fluorescently labeled UCMS–IFN-β cells (0.5 x 10^6, n=6) into non-tumor bearing mice. All the mice were sacrificed after 30 days by cervical dislocation. Lung weights were measured to estimate tumor burden. Lung, spleen, liver, kidney, and bone marrow were harvested and subjected for histological analysis.

Statistical analysis

To evaluate the significance of overall differences in lung weights between all *in vivo* groups, statistical analysis was performed by ANOVA. A p-value less than 0.05 were considered as significant. Following significant ANOVA, post hoc analysis using Fisher's protected least significance difference (PLSD) was used for multiple comparisons. Significance for post hoc testing was set at $p < 0.05$. All the lung weight data were represented as Mean ± Standard Error on graphs. Statistical analyses were performed by Stat View software, version 5.0.1. (Cary, NC).

Results

Secretion of IFN-β by UCMS-IFN-β cells

The amount of IFN-β in international units (I.U.) secreted by the UCMS–IFN-β cells into the media was measured by IFN-β ELISA kit. IFN-β was secreted by the UCMS–IFN-β cells in significant amounts, and there was a trend for more IFN-β release into the media when UCMS cells were transduced with a greater number of viral particles (VP) per cell (Table 4.1). IFN-β was not detected in the conditioned media with UCMS cells (data not shown).

Transduction: Number of viral particles/cell	Total amount IFN-β secreted by 10^6 UCMS–IFN-β cells in Pico grams	Total amount secreted by 10^6 UCMS–IFN-β cells in international units
12,500	929364.7	37175 ± 7548
6,400	692657.2	27706 ± 1022
3,200	509227.3	20369 ± 3958

Table 4.1 IFN-β (international units) secreted by $1x10^6$ human UCMS–IFN-β cells when transduced with 12500, 6400 and 3200 IFN-β adeno viralparticles/cell.

In vitro effect of IFN-β secreted by human UCMS-IFN-β cells on MDA 231 cells

To investigate whether human UCMS–IFN-β cells have an effect on growth and cell viability on MDA 231 cells, conditioned media from human UCMS–IFN-β cells was added to MDA 231 cells and its effect was analyzed. MDA 231 cell proliferation was markedly inhibited when cultured with conditioned media from UCMS–IFN-β cells in comparison with MDA 231 cells cultured in unconditioned UCMS media ($p<0.001$, Figure 4.1B). The percentage of dead MDA 231 cells in cultures with conditioned media from UCMS–IFN-β cells was significantly higher than in MDA 231 cells cultured in unconditioned UCMS media ($p<0.001$, Figure 4.1A).

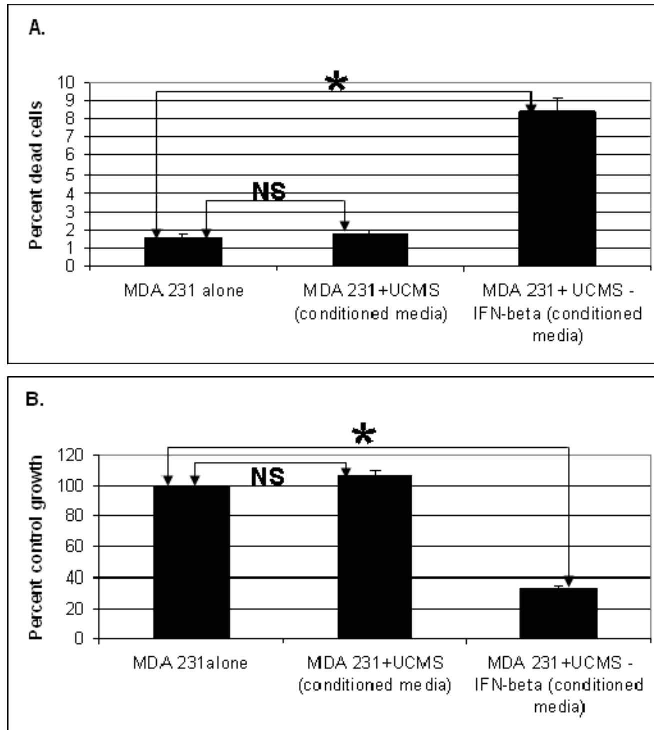

Figure 4.1 *In vitro* effect of human UCMS cells and human UCMS–IFN-β cells conditioned media on MDA 231 cells.

(A): Percentage dead cells and (B): percentage control cell growth of MDA 231 cells when treated with UCMS cells and UCMS–IFN-β cells conditioned media. * Statistically significant in comparison with MDA 231 only (p-value < 0.001)

Human UCMS cells do not form tumors in SCID mice

All the mice were sacrificed 50 days after UCMS cells injection. Mice appeared healthy and no clinical symptoms were found throughout the experimental period. No gross visible tumors were found around the transplantation sites or in any other regions of mice. Immunohistochemical analysis using serial sections stained by anti-human mitochondrial antibodies also failed to detect evidence of tumors in tissues such as

kidney, liver, and lungs (Figure 4.2 A, B, and C). These results suggest that human UCMS cells are non-tumorigenic in SCID mice and thus are potentially usable for transplantation studies.

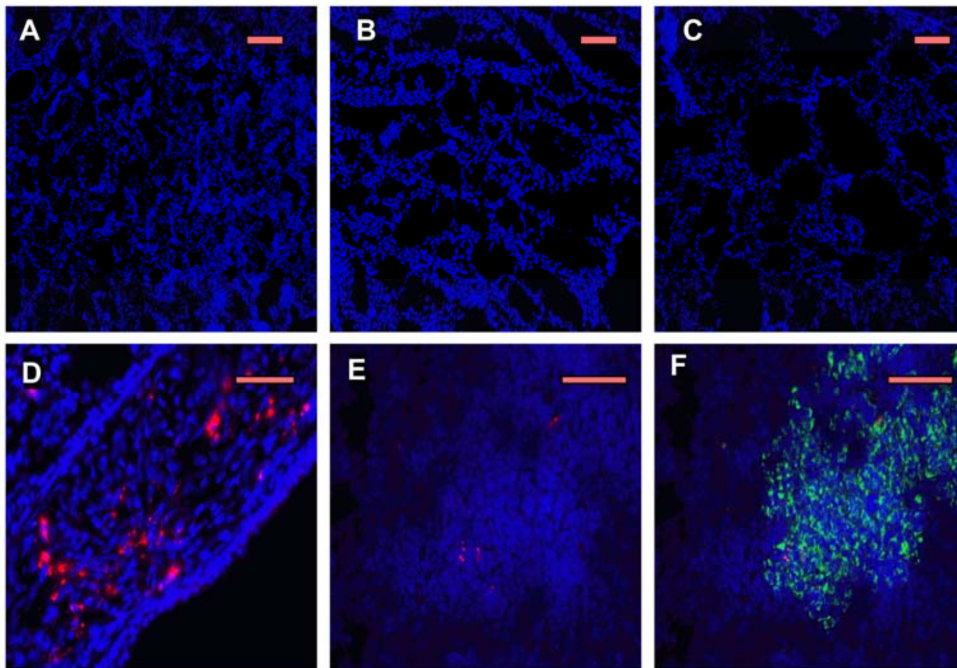

Figure 4.2 Absence of tumor formation in SCID mice injected with human UCMS cells. A: Kidney section negative for UCMS cells after immunostaining with anti-human mitochondrial antibody and Hoechst 33342 nuclear stain (blue) (B): Cross section of liver and (C): lung stained with same antibody. (Scale bar =100μm). (D-F): Selective engraftment of fluorescently labeled human UCMS cells in MDA 231 lung tumors counterstained with Hoechst 33342 nuclear stain. D and E: Fluorescent micrograph showing red labeled human UCMS cells (SP-DiI) counter stained with Hoechst 33342 nuclear stain (blue) and figure 4.2F is the same section as E but with tumor cells shown as green fluorescing cells after detection with antihuman mitochondrial antibody. (Scale bar =100μm).

Engraftment of human UCMS cells in MDA 231 xenografts in lungs of SCID mice

Red fluorescent human UCMS cells were found selectively near or within MDA 231 xenografts in lungs of SCID mice after intravenous transplantation (Figure 4.2 E and F). In contrast, in mice injected with MDA 231 cells alone, no red-fluorescent cells were found near or within the tumors (data not shown). Similarly, in mice injected with UCMS cells alone, and mice with PBS alone, no fluorescent cells were found in the lungs. These results suggest that UCMS cells are capable of tumor-targeted migration and survival in the tumor microenvironment. Lung weights from non-tumor bearing mice that received fluorescently labeled UCMS cells alone were not different than mice injected with PBS. As reported there was significant difference ($p<0.05$) in lung weights in mice that received MDA 231 cells than the mice that did not receive any MDA 231 cells (Figure 4.3).

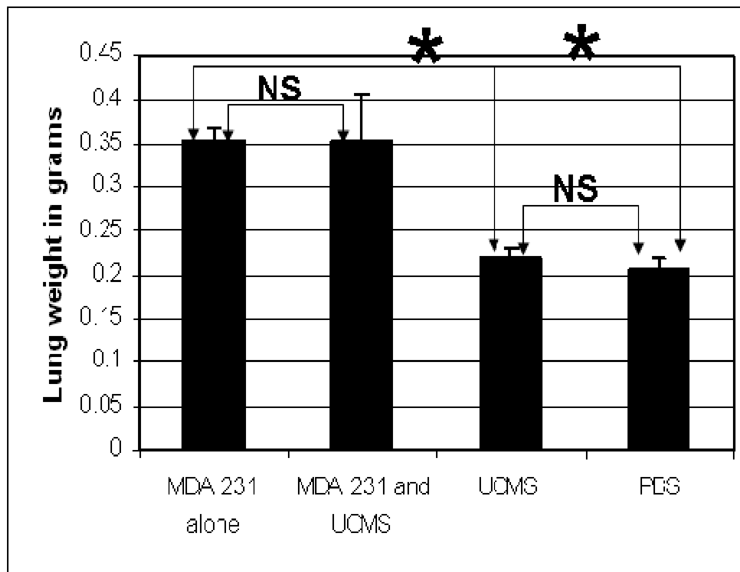

Figure 4.3 Effect of human UCMS cells (not expressing IFN-β) on tumor burden.

Lung weights of mice injected with only MDA 231 cells, MDA 231 and UCMS cells, UCMS cells alone (sham MDA 231 transplant), and PBS alone (sham tumor, sham transplant) are shown. * Statistically significant in comparison with mice with MDA 231 only (p-value < 0.05)

Lungs from tumor bearing mice, not UCMS cell transplanted were not different from tumor bearing mice transplanted with fluorescently labeled UCMS cells, indicating there was no significant effect of UCMS cells on tumor burden (Figure 4.3). In the lungs of mice injected with MDA 231 cells and fluorescently labeled UCMS cells, a large number of fluorescently labeled UCMS cells were found near or within tumors (Figure 4.2 E, and F) but were not found in kidney, liver or spleen.

Effect of UCMS-IFN-β cells on MDA 231 lung tumor burden in SCID mice

To test the hypothesis that UCMS–IFN-β cells would reduce the tumor burden in lungs of mice injected with MDA 231 cells, lung weights were used to estimate of tumor burden and lungs from various groups were photographed (Figure 4.4).

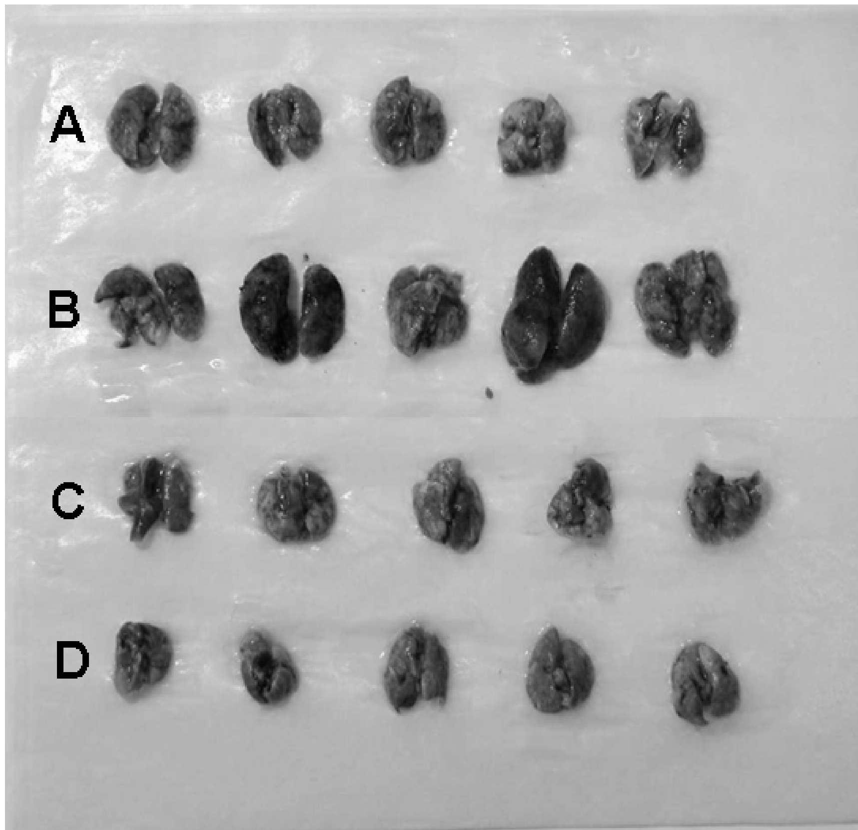

Figure 4.4 Representative lungs of experimental groups.

Group A. MDA 231+UCMS-IFN-β, Group B. MDA 231 alone, Group C. UCMS-IFN-β cells, no tumor cells, and Group D. PBS alone

Tumor burden was significantly reduced in MDA 231 inoculated mice transplanted with UCMS–IFN-β cells compared with mice inoculated with MDA 231 cells alone ($p<0.01$). In fact, the burden was reduced by about 50% (Figure 4.5). Lungs weights from mice that received only UCMS–IFN-β cells but not MDA tumor cells were not different than sham-treated mice (non significant (NS), $p>0.05$). Mice injected with UCMS–IFN-β cells alone did not show any adverse effects. Histological analysis revealed that engraftment of UCMS cells was detected in close proximity or within lung

tumor tissues of tumor bearing mice that either received fluorescently labeled UCMS–IFN-β cells or fluorescently labeled un-engineered UCMS cells (Figure 4.2 D). No fluorescently labeled UCMS cells were found in other tissues such as liver, spleen, and kidney.

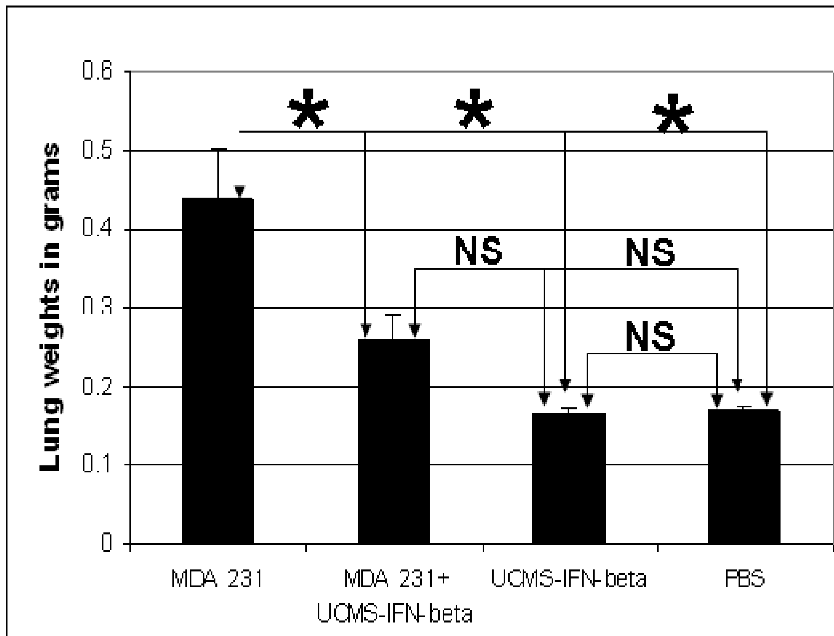

Figure 4.5 Comparative Lung weights.

Lung weights of mice which were injected with both MDA 231 and UCMS–IFN-β cells compared with mice injected with MDA 231 alone, UCMS–IFN-β cells alone and sham transplanted mice with no tumors. * Statistically significant in comparison with mice with MDA 231 only (p-value < 0.01).

Discussion

Here, we provide evidence that unique primitive human stem cells can be harvested rapidly in large numbers from umbilical cord Wharton's jelly do not themselves form tumors, and thus they are safe, practical delivery vehicles for specific

genes. For the first time, we show that UCMS cells selectively engraft in tumors, can be engineered to secrete a therapeutic protein, IFN-β, and significantly reduce lung metastasized breast carcinoma tumor burden in an animal model.

When undifferentiated embryonic stem cells (ESCs) are transplanted they sometimes form tumors (Thomson and Marshall, 1998;Wakitani et al., 2003;Arnhold et al., 2004;Ishikawa et al., 2004); therefore, we tested whether UCMS cells might have that tendency. UCMS cells are karyotypically stable over passages and do not lose anchorage dependency, contact inhibition or serum dependence (Weiss et al., 2006;Karahuseyinoglu et al., 2007), which are characteristics of cancer cells. Our *in vivo* findings support the *in vitro* published data, since they do not become tumorigenic and hence are safe from that standpoint. This is an important consideration, since these cells express some of the same primitive genes expressed by ESCs (Carlin et al., 2006).

IFN-β induces apoptosis in cancer cells mainly by disrupting mitochondria and activation of the caspase cascade (Juang et al., 2004). It also is a potent inhibitor of proliferation for many cancer cells *in vitro* (Wong et al., 1989;Johns et al., 1992). However, it cannot be used effectively as cancer therapy because the maximum tolerated dose is not high enough to attain these effects when it is given systemically (Salmon et al., 1996;Einhorn and Grander, 1996;Buchwalder et al., 2000) and it has a short half-life. Although it has been shown that serum levels of interferon delivered by engineered stem cells are insignificant in contrast to interferon given systemically (Studeny et al., 2004), the local production of IFN-β appears to be the key for tumor growth attenuation. The stem cell-based gene therapy can also avoid potential

systemic adverse effects of IFN-β. Since we found no long term engraftment of UCMS cells in the evaluation of tumor formation by stem cells (Figure 4.2 A-C), it is conceivable that the engrafted stem cells will not remain for a long period of time after attenuation of the tumor. In more recent work, we have engineered the UCMS cells with a thymidine kinase suicide gene as an additional safety feature (unpublished data). Taken together, our UCMS cell-based tumor-targeted IFN-β gene delivery appears to be a safe, effective, and practical cancer gene therapy.

The adenoviral system used to engineer the UCMS cells was fiber-modified to facilitate viral entry into the cells, since some cells may not express the coxsackie virus and adenovirus receptor (CAR) receptor necessary for cell entry (Dmitriev et al., 1998). Our *in vitro* testing of this fiber-modified vector established that it was considerably more efficient than transduction with unmodified adenoviral vectors (data not shown); this is consistent with other reports (Dmitriev et al., 1998;Mizuguchi et al., 2001;Koizumi et al., 2001).

The finding that UCMS cells administered via the tail vein exhibited a selective engraftment to the MDA 231 lung tumors of SCID mice suggests that they may respond to chemotactic signals emanating from the tumor cells. Others have reported that tumors secrete factors that recruit stromal, vascular, bone marrow and other stem cells to the tumor; theoretically the recruited cells provide a scaffolding and source of nutrients (Tlsty and Hein, 2001;van Kempen et al., 2003). Several chemokines are known to be secreted by tumors that may mediate the observed tropism, including VEGF, TGF family members, FGF family members, PDGF family members, MCP-1, EGF and IL-8 (Nakamura et al., 2004). Cells related to UCMS cells, bone marrow

MSCs, have been shown to exhibit a tropism for damaged or rapidly growing tissues as well as tumors (Azizi et al., 1998;Kopen et al., 1999;Studeny et al., 2002;Studeny et al., 2004;Nakamizo et al., 2005). The human UCMS cells have been characterized by gene array and cytokine array previously (Weiss et al., 2006), and express possible chemokine receptors such as SDFR1, TGFBR3, FGFR2 (Weiss et al., 2006).

In summary, human UCMS cells are unique stem cells derived from the Wharton's jelly of the umbilical cord and do not form tumors in SCID mice. Second, they selectively engraft in MDA 231 lung tumors. Third, UCMS cells that were engineered to express IFN-β can deliver sufficient IFN-β to kill MDA 231 cells *in vitro*. Finally, UCMS–IFN-β cell transplantation into lung tumor-bearing SCID mice produces a significant reduction of the tumor burden. Thus, these results suggest that UCMS cells may be a platform for targeted delivery of therapeutic proteins to human breast cancer and other types of cancer.

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CHAPTER 5 - Combination treatment of human umbilical cord matrix stem cell-based interferon-beta gene therapy and 5-fluorouracil significantly reduces growth of metastatic human breast cancer in SCID mouse lungs

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Key Words. Interferon-beta; lung tumors; UCMS cells; MDA-231 breast carcinoma cells; 5-fluorouracil.

Abstract

Umbilical cord matrix stem (UCMS) cells that were engineered to express interferon-beta (IFN-β) were transplanted weekly for three weeks into MDA 231 breast cancer xenografts bearing SCID mice in combination with 5-fluorouracil (5-FU). The UCMS cells were found within lung tumors but not in other tissues. Although both treatments significantly reduced MDA 231 tumor area in the SCID mouse lungs, the combined treatment resulted in a greater reduction in tumor area than by either treatment used alone. These results indicate that a combination treatment of UCMS-IFN-β cells and 5-FU is a potentially effective therapeutic procedure for breast cancer.

1. Introduction

Tumors behave much like wounds that do not heal (Dvorak, 1986). Bone marrow mesenchymal stem cells (MSCs) (Natsu et al., 2004;Rojas et al., 2005) and neural stem cells (Aboody et al., 2000;Brown et al., 2003) migrate toward wounds and other areas of pathology and have been shown to be effective gene-delivery vehicles for targeted cancer therapy (Aboody et al., 2000;Studeny et al., 2002;Brown et al., 2003;Studeny et al., 2004;Nakamura et al., 2004). However, bone marrow cells require painfully invasive procedures to harvest and require considerable time for expansion. On the other hand, umbilical cord matrix stem (UCMS) cells, isolated from Wharton's jelly of the umbilical cord (Mitchell et al., 2003), are unique in that they are prenatal cells isolated postnatally, and in contrast to many postnatal stem cells, can be harvested non-invasively in large numbers. Gene array and RT PCR data indicate that UCMS cells express genes expressed by undifferentiated embryonic stem cells (ESCs),

including those of the LIF receptor pathway, ESG1, SOX2, Oct4, Nanog and TERT (Weiss et al., 2006;Carlin et al., 2006). Human UCMS cells express markers of three germ layer derivatives: mesoderm: ACTG2, ACTA2, BMP1, PDGFB; ectoderm: cytokeratin, SHH; and endoderm: insulin. Given their primitive nature, it is worrisome that UCMS cells might tend to form teratomas, as is the case with ESCs (Thomson and Marshall, 1998;Wakitani et al., 2003;Arnhold et al., 2004;Ishikawa et al., 2004). However, they do not become tumorgenic after multiple passages (Karahuseyinoglu et al., 2007) or after transplantation of large numbers into SCID mice (Rachakatla et al., 2007).

The cytokine interferon-beta (IFN-β) is known to have multiple effects such as immunostimulation, inhibition of tumor growth and angiogenesis (Sidky and Borden, 1987;Singh et al., 1995;Qin et al., 1997;Stark et al., 1998;Qin et al., 1998). In an earlier experiment, we found that UCMS cells engineered to secrete IFN-β trafficked to and reduced the tumor burden of lung tumors in SCID mice (Rachakatla et al., 2007). Here, we investigated whether further reduction of tumor burden could be effected by increasing the expression of IFN-β by the UCMS cells and by using this targeted delivery in combination with a low dose of 5-Fluorouracil (5-FU). 5-FU is a widely used chemotherapeutic agent that often has significant hematological and digestive toxic side effects (Douillard et al., 2000); however, those side effects are substantially reduced at a lower dose. Choi *et al* have shown that combination of adenoviral mediated IFN-β gene therapy and 5-FU have reduced tumors and improved the survival rates of liver metastatic tumor bearing mice models (Choi et al., 2004). Accordingly, our hypothesis was that stem cell mediated targeted delivery of IFN-β would allow the use of reduced

dosages of 5-FU. Here, we report for the first time that stem cell-mediated gene delivery of IFN-β, in combination with systemic treatment of low doses of 5-FU, results in significant additive effects on tumor attenuation in a rodent model of metastatic breast carcinoma.

2. Materials and Methods

2.1. Tissue culture of human umbilical cord matrix stem cells and MDA 231 cells

Human umbilical cord matrix stem (UCMS) cells were harvested from term deliveries at the time of birth with the mother's consent. The methods to isolate and culture human UCMS cells were previously described (Weiss et al., 2006). Human UCMS cells were maintained in defined medium (DM), a mixture of 56% low glucose DMEM (Invitrogen), 37% MCBD 201 (Sigma; St. Louis, MO) and 2% fetal bovine serum (FBS, Atlanta Biologicals Inc, Georgia) containing 1x insulin-transferrin-selenium-X (ITS-X, Invitrogen, CA), 1x ALBUMax1 (Invitrogen, CA), 1x Pen /Strep (Invitrogen, CA), 10nM dexamethasone (Sigma, MO), 100μM ascorbic acid 2-phosphate (Sigma, MO), 10ng/ml epidermal growth factor (EGF, R&D systems, Minneapolis), and 10ng/ml platelet derived growth factor-BB (PDGF-BB, R&D systems, MN), at 37°C in a humidified atmosphere containing 5% carbon dioxide.

MDA 231 human breast carcinoma cells that metastasize to the lung in nude mice were obtained from M.D. Anderson Cancer Center (Houston, TX) (Studeny et al., 2002). They were maintained in DMEM (Invitrogen, CA), 1x Pen/Strep (Invitrogen, CA),

and 10% FBS (Atlanta Biologicals Inc, GA), at 37°C in a humidified atmosphere containing 5% carbon dioxide.

2.2. Transduction of human UCMS cells with recombinant IFN-β adenovirus vector and quantification of IFN-β

Since UCMS cells (like bone marrow MSC) do not express the adenoviral receptor, they were engineered using fiber-modified IFN-β recombinant adenovirus (Studeny et al., 2002), developed by one of us (F. M.). For transduction, the human UCMS cells were washed twice with DMEM without serum and cells were incubated with DMEM containing adenovirus at 100,000 plaque forming units (PFU) per cell for 4 hours at 37°C. Defined medium (DM) containing 5% FBS was added after incubation and the cells were used for *in vivo* transplantation the next day. The amount of IFN-β secreted by UCMS–IFN-β cells into the medium was quantified by a human interferon-beta ELISA kit (PBL Biomedical Laboratories, New Jersey). For quantification of IFN-β, UCMS cells were plated at 50,000 cells per well in a 12-well plate. UCMS cells were transduced with adenovirus expressing IFN-β at concentrations of 10,000, 25,000, 50,000, and 100,000 PFU per cell. The UCMS–IFN-β cells were cultured for 96 hours and media was collected at 24, 48 and 96 hours and stored at -80^{0}C until use. After collecting media at 3 different time points, ELISA assay was carried out according to manufacturer's protocols.

2.3. *In vitro* apoptotic effect of conditioned medium from UCMS-IFN-β cells on MDA 231 cells

To condition the media, fresh DM containing 5% FBS was added to sub-confluent (20,000 cells/cm^2) UCMS cells and UCMS-IFN-β cells and cultured for 48 hours (first 48 hours, 0-48 hours from transduction). The conditioned medium (CM) was removed and the cells refed and cultured for an additional 48 hours (second 48 hours, 48-96 hours from transduction). The CM was removed and stored at -80°C until use. The MDA 231 cells (0.25×10^6 cells/well) were cultured in 6-well plates with DM containing 5% FBS. Cells were then incubated until approximately 90% confluency was reached (approximately 48 hours), at which time the medium was changed to various types of DM-diluted conditioned media (CM, 1:1=DM:CM). The assay was carried out with quadruplicate determinations and was repeated at least twice. Following the medium change to the conditioned media, the cells were incubated for a period of 24 hours and their apoptotic index was analyzed. When cells had been treated as described above, they were prepared for Annexin V-FITC FACS analysis, according to manufacturer's protocol. The cells were first washed with PBS, trypsinized and resuspended in DM containing 5% FBS. Approximately 2×10^5 MDA 231 cells from various treatments were subjected to evaluation of apoptosis index by the Annexin V-FITC Apoptosis Detection Kit (Bio Vision Inc. Mountain View, CA). The FACS analysis was carried out by FACSVantage SE flow cytometer (BD Biosciences, San Jose, CA).

Additionally, we examined caspase activations after the combination treatment. MDA 231 cells were plated in 6-well plates at a density of 200,000 cells per well with DM containing 5% FBS. Twenty four hours later, the medium was removed from all the

wells and was replaced with either 5-FU (10μg, 100μg, 250μg/ml of DM), or DM-diluted CM from UCMS-IFN-β cells (second 48 hours conditioned medium) or a combination of both. The cells were allowed to incubate for 36 and 48 hours. Later, cells were lysed with lysis buffer (1% Triton,0.1%SDS, 0.25M sucrose, 1mM EDTA, 30mM Tris-HCl pH8.0 and 7x complete mini EDTA-free stock solution (Roche Diagnostics, IN) at ratio of 1:7) followed by sonication and boiling. After cells were lysed, 1/4 volume of 5x SDS-PAGE sample buffer was added to the cell lysis solution. Whole cell protein was separated with 12%SDS-PAGE and transferred to nitrocellusose membrane (GE Healthcare, NJ). The membrane was probed with a specific primary antibody (procaspase-3, cleaved caspase-3 (Cell Signaling Technology, MA)) and GAPDH (Santa Cruz Biotechnology, CA) at dilution of 1: 1000 to 1: 4000, followed by washing and probing with anti-rabbit HRP-antibody (GE Healthcare, NJ) at dilution of 1: 2000. The specific protein band was visualized by a Kodak image station using a super signal kit (Price Biotechnology, IL).

2.4. *In vitro* additive effect of 5-FU and UCMS-IFN-β cell conditioned medium on cell proliferation and viability of MDA 231 cells

MDA 231 cells were plated in 6-well plates at a density of 200,000 cells per well with DM containing 5% FBS. Twenty four hours later, the medium was removed from all the wells and was replaced with either 5-FU (10μg, 100μg, 250μg/ml of DM), or DM-diluted CM from UCMS-IFN-β cells (second 48 hours conditioned medium) or a combination of both. After 24 hours, the cells were trypsinized; cell proliferation and viability were quantified by MTT assay (Roche Applied Science, IN) as per

manufacturer's protocol. Three different isolates of UCMS cells were used in separate trials and each experiment was done in triplicate.

2.5. Identification of human UCMS cells and MDA 231 cells in mouse tissues

For transplanted stem cell identification, the fluorescent dye SP-DiI (Molecular Probes, CA) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 5mg/ml. SP-DiI dye was added to culture medium to a final concentration of 10μg/ml and human UCMS cells were labeled by adding 10 ml of medium with SP-DiI in a T-75 flask for 24 hours. Then, cells were washed with PBS, incubated with dye-free medium for 4 hours, and used for experiments. Immunohistochemistry was used to identify the MDA 231 cells in mouse lung. Tissue sections were washed with phosphate buffered saline-0.2% Triton X-100 (PBS TX) and fixed with 70% ethanol and acetone (1:1). This was followed by washing with three changes of PBS TX. To more clearly delineate tumor cells, tissue sections were blocked with 5% normal goat serum in PBS TX for 30 minutes, followed by incubation with anti-human mitochondrial antibody (1:100, Chemicon, CA), in PBS TX overnight. The tissues were then washed three times with PBS TX and incubated with Alexa Fluor 488 conjugated secondary antibody (1:1000, Molecular Probes, CA) for 3 hours. The tissues were incubated for 30 min. in Hoechst 33342 (10μg/ml, Sigma, MO) nuclear counterstain, followed by a triple rinse with PBS TX. The antigens were localized using epifluorescence microscopy (Nikon Eclipse, Boyce Scientific Inc. MO) and images were captured using a Roper Cool Snap ES camera and Metamorph 7.

2.6. Evaluation of the *in vivo* effect of 5-FU and human UCMS-IFN-β cells on MDA 231 tumors

Female CB-17 SCID mice (5 weeks old) were obtained from Harlan Laboratories (Indiana). Mice were held for 1 week after arrival to allow them to acclimate. MDA 231 cells (2 X $10^{6)}$ were transplanted into the lateral tail vein using sterile conditions. Eight days after tumor transplant, mice were randomized into five groups: 1) sham UCMS cell transplant; 2) UCMS–IFN-β cell (0.5 X 10^6) transplant; 3) un-engineered UCMS cell (0.5 X 10^6) transplant; 4) UCMS–IFN-β cell transplant and 5-FU (40mg/kg), given via intraperitoneal (IP) injection 3 days after each UCMS–IFN-β cell transplant); and 5) 5-FU given alone on days corresponding to group 4 injections. An additional group that received saline sham tumor cell and stem cell transplants served as untreated controls. All the mice were sacrificed 30 days after tumor inoculation by CO_2 inhalation and cervical dislocation. Lung weights of control and tumor-bearing animals were measured to estimate tumor burden (ANOVA followed by post-hoc planned comparison). Lungs were snap-frozen in isopentane in liquid nitrogen for histological and/or immunohistochemical (IHC) analysis. Other organs including spleen, liver, kidney, and bone marrow were also harvested. Tissues were sectioned on a cryostat at 7-8 μm and used for IHC studies. Random tissue sections from each group of SCID mice were taken to measure the tumor area in the lungs. Immunohistochemistry, performed as described above using anti-human mitochondrial antibody, served to outline the MDA 231 tumors with green fluorescence. The area (in square micrometers) occupied by the tumor was calculated from 10-15 200x fields for each group of mice using the MetaMorph 7 image analysis system as described elsewhere (Koka et al.,

2006;Richards et al., 2007). Several investigators have calculated random areas in a tissue section and have correlated it to overall tissue section using the MetaMorph 7 image analysis system (Koka et al., 2006;Richards et al., 2007).

2.7. Statistical analysis

The means of the experimental groups were evaluated to confirm that they met the normality assumption. For *in vitro* experiments, statistical analysis was performed with the student's t-test. To evaluate the significance of overall differences in tumor areas between all *in vivo* groups, statistical analysis was performed by ANOVA. A p-value less than 0.05 was considered as significant. Following significant ANOVA, post hoc analysis using Fisher's protected least significance difference (PLSD) was used for multiple comparisons. Significance for post hoc testing was set at $p < 0.05$. All the tumor area data were represented as mean ± standard error on graphs. Statistical analyses were performed by Stat View software, version 5.0.1. (Cary, NC).

3. Results

3.1. Quantification of IFN-β secreted by UCMS-IFN-β cells

The amount of IFN-β secreted by the UCMS–IFN-β cells into the medium was measured by ELISA kit (Table 1). IFN-β was secreted in significant amounts and there was a trend for more IFN-β release into the medium when UCMS cells were transduced with a greater number of plaque forming units. The time-dependent increase of IFN-β secretion was also observed when UCMS–IFN-β cells were incubated for 24, 48, and

96 hours (second 48 hour incubation after the medium change) after the IFN-β transfection (Table 5.1).

Adenoviral IFN-β (PFU/cell)	Total amount of IFN-β secreted by 10^6 UCMS–IFN-β cells (μg)	Total amount of secreted by 10^6 UCMS–IFN-β cells (I.U)
50,000	4.45 (24 hours)	178107 +/- 7643
100,000	9.34 (24 hours)	373642 +/- 4321
50,000	4.88 (first 48 hours)	195309 +/- 5320
50,000	8.07 (second 48 hours)	323136 +/- 2332

Table 5.1 The levels of IFN-β secreted by $1x10^6$ human IFN-β transfected UCMS cells were dose- and time-dependently increased.

Designated amounts (PFU/cell) of adenovirus vector containing IFN-β DNA were incubated with UCMS cells for 24, 48 and 96 hours. The level of IFN-β was quantified by ELISA as described in the methods section. Twenty four and 48 hour samples were obtained from the conditioned medium incubated with transfected cells for 24 and 48 hours after the transfection, respectively. The second 48 hour sample was collected from 48-96 hours after transfection.

3.2. Effect of UCMS–IFN-β cell conditioned medium (CM) on apoptosis of MDA 231 cells

To investigate the effect of UCMS–IFN-β cell CM upon apoptosis of MDA 231 cells, the MDA 231 cells were cultured with CM from UCMS cells and UCMS–IFN-β cells for several time intervals. The percentage of MDA 231 cells in late apoptosis significantly increased in cells cultured with both first 48 hours (data not shown) and

second 48 hours UCMS–IFN-β cell CM (Figure 5.1) in comparison with MDA 231 cells cultured with unconditioned DM or UCMS cell CM (i.e., since the 0-48 hr CM from UCMS–IFN-β showed a similar effect on the tumor cells as the 48-96 hr interval, only the latter is shown). The percentage of cells in early apoptosis in the MDA 231 cells cultured with UCMS–IFN-β cell CM was significantly higher than unconditioned DM group (Figure 5.1).

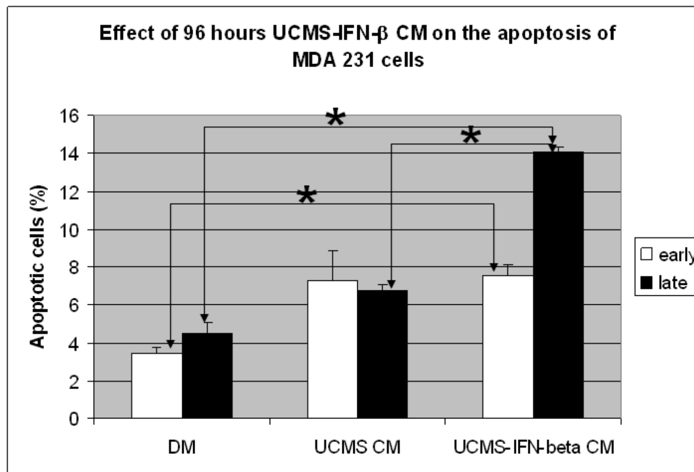

Figure 5.1 *In vitro* apoptotic effect of UCMS-IFN-β cell conditioned medium on MDA 231 cells.

Percentage of cells in early and late apoptosis when treated with second 48 hours CM (48-96 hours from the transduction). * Statistically significant (t-value less than 0.05).

The conditioned medium obtained from non-interferon-expressing UCMS cells showed some effect on cancer cell apoptosis in this study; however, the IFN-β transfected UCMS-derived conditioned medium always exhibited a stronger apoptotic effect than the conditioned medium obtained from the UCMS cells alone in the parallel study.

Western blot results showed that procaspase-3 is decreased and activated caspase-3 is increased in combination therapy of 5-FU and UCMS-IFN-β cells (Figure 5.2).These results clearly indicate that the combination treatment of UCMS–IFN-β cells and 5-FU decreased cell viability and increased the apoptosis more effectively than individual treatment.

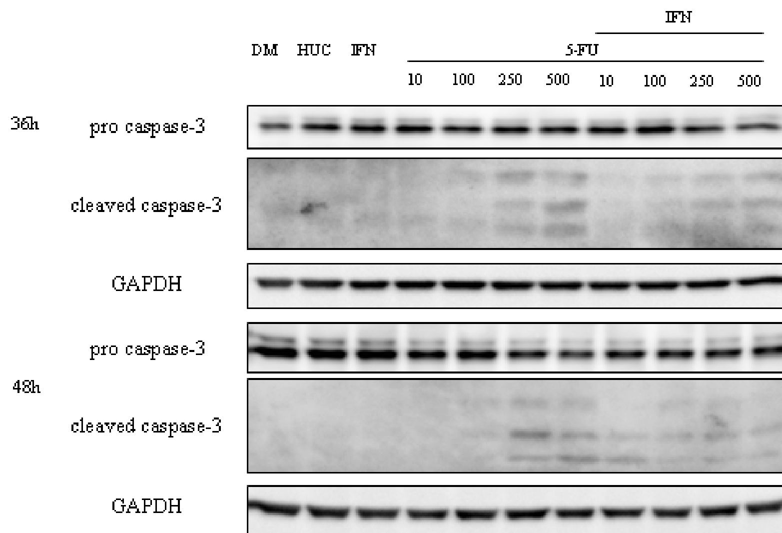

Figure 5.2 Western blot analysis of caspase 3 activation in MDA 231 cells treated with 5-FU and the conditioned medium with UCMS-IFN-β cells.

In this experiment, a combination treatment with 5-FU and the conditioned medium significantly stimulated an activation of caspase 3. Note that the treatment with low concentration (10 and 100 μg) of 5-FU and conditioned medium significantly increased band intensities of the cleaved caspase 3 in a time-dependent manner.

3.3. Effect of 5-FU and UCMS–IFN-β cell conditioned medium on MDA 231 cell proliferation and viability

To investigate the effect of 5-FU and UCMS–IFN-β cell CM on proliferation and viability of MDA 231 cells, 5-FU (10μg, 100μg, 250μg/ml of DM) or UCMS-IFN-β cell CM or a combination of both were added to the MDA 231 cells and cultured for 24 hours. The viability of the MDA 231 cells was significantly decreased in a dose-dependent manner by addition of either 5-FU or UCMS-IFN-β cell CM or combination of both in comparison with cells cultured in DM containing 5% FBS. There was an additive effect when MDA 231 cells were cultured both in 5-FU and UCMS-IFN-β cell CM compared to when they were cultured in either 5-FU or UCMS-IFN-β cell CM alone (data not shown).

3.4. Effect of UCMS–IFN-β cells and 5-FU on MDA-231 lung tumor burden in SCID mice

Lung tumor burden was significantly reduced in MDA 231 inoculated mice transplanted with UCMS–IFN-β cells compared with mice inoculated with MDA 231 cells alone ($p<0.01$)(Figure 5.3).

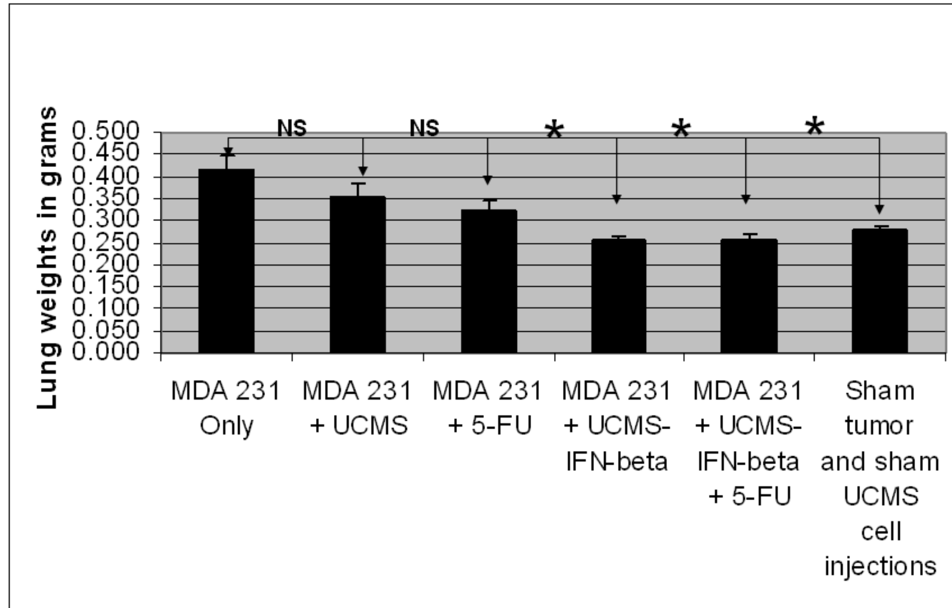

Figure 5.3 Comparative Lung weights.

Lung weights of mice which were injected with MDA 231 alone compared with mice which were injected with both MDA 231 plus UCMS cells or 5-FU or UCMS–IFN-β cells or combination of both UCMS–IFN-β cells and 5-FU. Mice injected with sham tumor and sham UCMS cell injections served as untreated controls. * Statistically significant in comparison with mice with MDA 231 only (p-value < 0.01)

Lung weights from mice that received either MDA 231 tumor cells and UCMS–IFN-β cells or mice that received MDA 231 tumor cells, 5-FU and UCMS–IFN-β cells were not different than control animals (sham tumor and sham UCMS cell injected mice). Histological analysis revealed that engraftment of UCMS cells was detected in close proximity or within lung tumor tissues of tumor bearing mice that either received SP-DiI labeled UCMS–IFN-β cells or SP-DiI labeled un-engineered UCMS cells (Figure 5.4 B, D, and E).

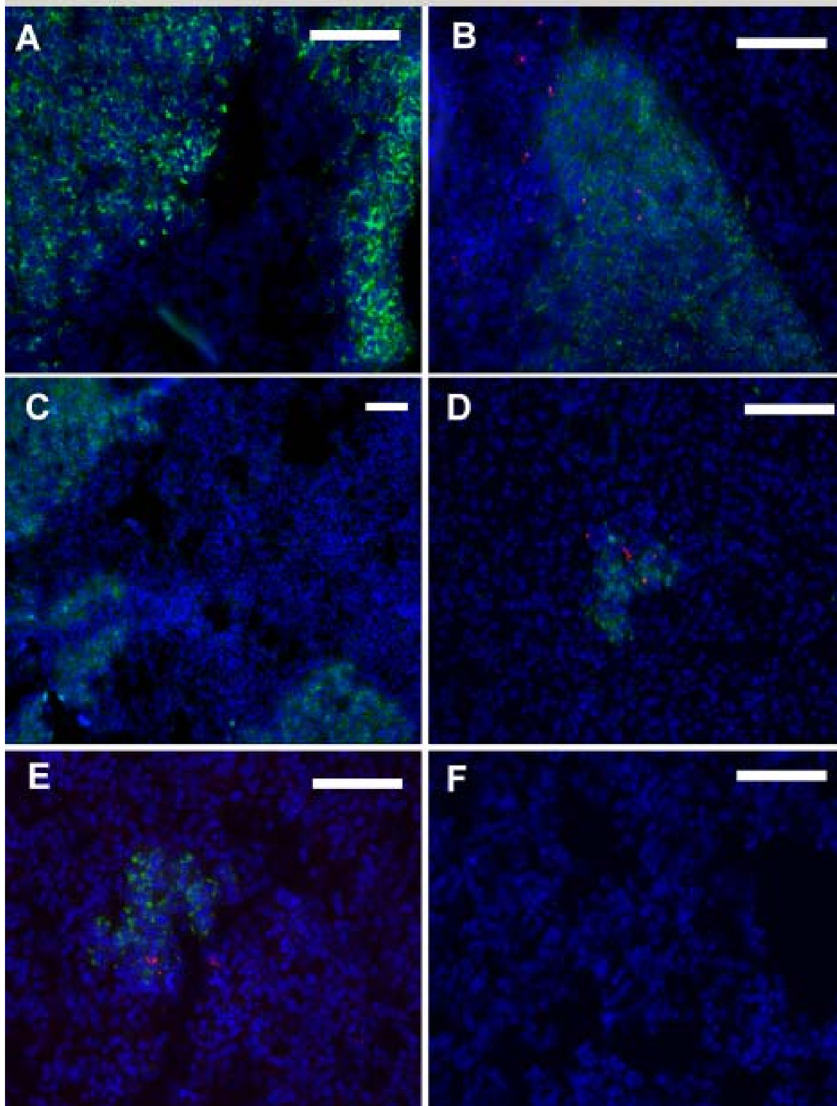

Figure 5.4 Selective engraftment and therapeutic effect of human UCMS-IFN-β cells in combination with 5-FU on MDA 231 lung tumors in SCID mice.

A: Fluorescent micrograph showing human mitochondrial stained MDA 231 cells (green) counterstained with Hoechst 33342 nuclear stain (blue) in mouse lung injected with MDA 231 cells alone. B: Lung section of mouse injected with MDA 231 cells and UCMS cells, showing selective engraftment of SP-DiI labeled UCMS cells (red) in close proximity to the MDA 231 lung tumors (green). C: Lung section of mouse injected with MDA 231 cells and 5-FU. D and E: MDA 231 tumor regression and engraftment of

UCMS cells in lung sections of mice injected with both MDA 231 cells and UCMS-IFN-β cells (D) and in combination with 5-FU (E). F: Saline control (sham tumor and sham UCMS cell injections) lung section negative for UCMS cells after immunostaining with anti-human mitochondrial antibody and Hoechst 33342 nuclear stain (blue). (Scale bar =100μm).

No SP-DiI labeled UCMS cells were found in other tissues, such as liver, spleen, and kidney. Tumor area was significantly reduced in all the groups that received either UCMS cells, 5-FU, UCMS–IFN-β cells, or both 5-FU and UCMS–IFN-β cells in comparison with mice that received only MDA 231 cells (Figure 5.5). Mice that received combined treatment of 5-FU and UCMS–IFN-β cells showed a greater reduction in tumor area than the mice that received either 5-FU or UCMS-IFN-β cells (Figure 5.5). These results indicate that this combination treatment most effectively reduced the tumor burden.

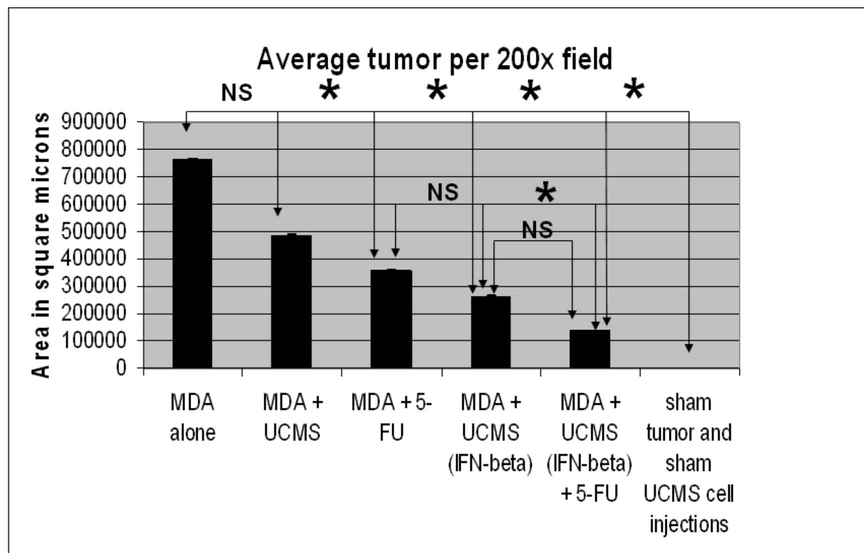

Figure 5.5 Combined effect of 5-FU and UCMS-IFN-β cells on tumor burden.

Graph depicting average tumor areas per square 200X field (lung sections) of MDA 231 tumor bearing mice which were later injected with UCMS cells, UCMS-IFN-β cells, 5-FU, or combined injections of 5-FU and UCMS-IFN-β cells. * Statistically significant in comparison with mice with MDA 231 only (p-value < 0.05).

4. Discussion

Here, we provide evidence that 1) *in vitro* treatment of MDA 231 cells with UCMS–IFN-β cell CM significantly increased both early and late apoptosis, and the combination of UCMS–IFN-β cell CM and 5-FU showed the greatest reduction in aviability of MDA 231 cells; 2) in SCID mice bearing lung tumors due to an inoculation of breast adenocarcinoma cells, UCMS cells selectively engraft in tumor tissue; 3) systemic administration of UCMS–IFN-β cells or 5-FU to the tumor-bearing SCID mice significantly reduced tumor burden; and 4) there was additive tumor attenuation after combined low-dose 5-FU and UCMS–IFN-β cells.

To our knowledge, this is the first report to demonstrate that efficacy of combining systemic chemotherapeutic treatment with stem cell-based-targeted delivery of a cytokine to a malignant human breast carcinoma in the lungs of SCID mice. The rationale for the combination is that possible toxic side effects are reduced in three ways: 1) the targeted delivery of an otherwise toxic cytokine by stem cells circumvents many of the effects it would cause if given systemically; 2) it may allow lower doses of a chemotherapeutic agent, which is better tolerated by patients; and 3) the two treatments may enhance each other. The dose of 5-FU used here was significantly below the

maximal tolerated dose of about 200 mg/kg for mice (Klubes and Cerna, 1983) and the 5-FU was only administered three times during weekly treatments.

IFN-β exhibits pleiotropic activities, including immunomodulatory and anti-tumor effects (Morris and Zvetkova, 1997;Gresser, 1997). It stimulates nitric oxide synthesis, which in turn inhibits growth and metastasis of some cancer cells (Wang et al., 2001). It activates caspases 3 and 8 (Kito et al., 1999), and induces TRAIL (tumor necrosis-related-apoptosis-inducing-ligand) synthesis (Morrison et al., 2002). Interferon-stimulated genes include XAF 1 (X-linked inhibitor of apoptosis associated factor-1), which increases the sensitivity of cells to TRAIL induced apoptosis (Leaman et al., 2002), and IRF5 (interferon regulatory factor 5), which sensitizes p53-deficient tumors to DNA damage-associated apoptosis (Hu et al., 2005). IFN-β induces $p27^{kip}$ cyclin-dependent kinase inhibitor and increases phosphorylation of the retinoblastoma gene, both of which inhibit proliferation of cancer cells (Kuniyasu et al., 1997). It inhibits angiogenesis by down-regulating basic fibroblast growth factor (Wang et al., 2001). Interferon-beta can also inhibit the effect of other growth factors (Einat et al., 1985).

We have carried out multiple *in vitro* experiments (cell counting, flow cytometry and caspase activation) in order to evaluate the effect of CM from UCMS-IFN-β cells, 5-FU, or combination of both on MDA 231 cells. Our data clearly indicate that IFN-β transfected UCMS cells secreted significant amounts of INF-β (Table1) and induced apoptosis of the cancer cells (Figure 1 and 2). Our data also indicated that a combination treatment of 5-FU and UCMS-IFN-β CM stimulated activation of caspase 3 at a low dose of 5-FU (Figure 2). Although, we did not perform experiments with anti-IFN-β antibody for the *in vitro* apoptosis studies, significantly increased IFN-β levels in

the CM suggest that IFN-β produced functionally stimulated cancer cell apoptosis. Our results are supported by several other studies in which IFN-β had an apoptotic and growth inhibitory effect on MDA 231 cells (Gibson et al., 1993). CM obtained from untransfected UCMS cells also increased early apoptosis index in flow cytometry analysis of annexin V (Figure 1). This may suggest that UCMS cell alone may produce a factor(s) that stimulate cancer cell apoptosis. However, the mechanism by which UCMS cell alone stimulate apoptosis must be addressed by separate study.

Tumors secrete factors that recruit cells from surrounding tissue as well as from the bone marrow to provide support and nutrition (Tlsty and Hein, 2001;van Kempen et al., 2003). Nakamizo *et al.* showed that SDF1 alpha, EGF and PDGF but not basic FGF or VEGF, enhanced bone marrow MSC migration to tumor cells (Nakamizo et al., 2005). Breast cancer cells have been reported to make the chemokines CCL5 and CCL2 that could cause stem cells to traffic to them (Kulbe et al., 2004). A therapeutic strategy using gene-transfected MSC as cellular vehicles for targeted gene delivery circumvents the problem of short life or excessive toxicity of cytotoxic cytokine(s) *in vivo*. IFN-β, for example, could not have been used effectively as a therapeutic agent because the maximum tolerated dose is not high enough to attain these effects when given systemically (Studeny et al., 2002). Studeny *et al.* demonstrated that injection of IFN-β transfected MSCs suppressed tumor growth and prolonged mouse survival without an acute increase in blood IFN-β concentration (Studeny et al., 2002).

The fact that large numbers of UCMS cells can be isolated rapidly is an advantage, allowing therapy to be initiated with minimal delay. This is particularly crucial for cancer types for which there is such a short life expectancy that the 'window'

for any given treatment may be quite small (Cohenuram and Saif, 2007). Therefore, UCMS cells engineered to secrete a cytokine such as IFN-β may be a future tool for targeted gene therapy of human breast and other cancers via stem cells with or without low doses of chemotherapeutic agents such as 5-FU.

Un-engineered UCMS cells also reduced the tumor burden in tumor-bearing SCID mice and *in vitro* data showed that CM from UCMS cells reduced the MDA 231 cell viability and proliferation. These results suggest that UCMS cells alone are capable of reducing growth of breast carcinoma cells. Further studies are needed to explain this effect.

Acknowledgments

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