Human Embryonic-like ECM (hECM) Stimulates Proliferation and Differentiation in Stem Cells While Killing Cancer Cells

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Background and Objectives: There are a number of unique processes seen in the developing fetus that cease post-partum including that tumors rarely form, and scar-less wound healing and digit regeneration occur. In addition, cancer lines have been “reprogrammed” by co-culture with embryonic extracellular matrix (ECM).

Methods and Results: We have developed a naturally secreted human ECM (hECM) with embryonic-like characteristics which is secreted by neonatal fibroblasts grown in microcarrier suspension cultures under hypoxia. This upregulates a number of substances associated with stem cell niches in the body including various laminins, Collagen 4, CXCL12, NID1, NID2, and NOTCH2. hECM has been shown to support proliferation of hESCs and MSCs and diminish or eliminate tumor load in melanoma (B16), adenocarcinoma (MDA-MB-435), colon cancer (HT29) and glioma (C6) in both *in vitro* and *in vivo* animal studies. In the tumor chorioallantoic membrane (tumcam) model hECM significantly inhibited tumor growth and in subcutaneous mouse xenograft experiments, tumor growth was inhibited from 70~90%. Co-cultures of fibroblasts and mesothelioma show support of fibroblast expansion with a concurrent inhibition of mesothelioma. The inhibitory affect is selective for cancer cells and cancer stem cells through the upregulation of Caspase 9 which forces the cells into apoptosis.

Conclusions: These data show that hECM has the potential to show benefit in the treatment of various cancers as a coating for biopsy needle, tissue filler post tumor removal, and as an injectable into the tumor site.

Keywords: Stem cells, Cancer, Extracellular matrix, Embryonic ECM, Tissue-engineered ECM

Introduction

The tumor microenvironment consists of laminin, collagen, and other diverse extracellular matrix proteins as well as growth factors and nutrients which interact with the malignant cells. Recent studies have shown that the embryonic microenvironment has the ability to normalize aggressive melanoma cells to a melanocyte phenotype (1). In addition, the embryonic condition of hypoxia has been

shown to be important for melanocyte transformation, and melanoma cells assume a neural crest cell-like phenotype in an embryonic microenvironment (2). The embryonic environment is known to be characterized by rapid cell growth and scarless healing (3). The embryonic tissue is hypoxic, and, unlike adult tissue, contains predominantly collagens III and V and low levels of TGF β and bFGF. Although traditional mammalian cell culture has involved normoxic conditions of 21% oxygen and 5% CO₂, Ezashi et al. (4) has shown that mimicking the embryonic condition of hypoxia enhances the growth of human embryonic stem cells (hESCs) and both hESCs and adult stem cells show improved maintenance of their undifferentiated state under low oxygen conditions (5-7). Given these findings on how the microenvironment can effect normal and malignant cell growth and differentiation, we sought to examine the effects of soluble and insoluble extracellular

Accepted for publication March 13, 2011

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matrix components (ECM) secreted by cells grown under hypoxia on normal and malignant cell growth.

Materials and Methods

Neonatal human fibroblasts derived from foreskin surgical discards were isolated and passaged to allow a cell expansion sufficient to establish a Master Cell Bank (8). Cells were tested for all known pathogens and virus as established by CBER's *Points to Consider* (9). Cells were seeded on dextran beads and grown under 1~5% oxygen in tightly controlled stirred bioreactor tanks. Cells were removed periodically from the manufacturing system and cellular RNA was isolated using a SV Total RNA kit (Promega) and subsequently analyzed for changes in gene expression using Agilent whole human genome microarrays. In addition, cells grown in hypoxic conditions were formalin fixed and immunocytochemically stained using primary antibodies to stem cell-associated markers that were detected using Alexa-488 or HRP-conjugated secondary antibodies.

Conditioned media was removed over a twelve week period and assayed for key growth factors and morphogens by ELISA. At the end of the manufacturing period all conditioned media was removed and lyophilized and then

brought to a slurry state by the addition of minimal amounts of sterile water. The cells on the beads were lysed with sterile water and beads were digested with dextranase in order to isolate the insoluble ECM proteins secreted. Western blot analysis was performed to help characterize the individual secreted proteins.

A series of *in vitro* studies were performed to assess the effects of petri dishes coated with either soluble or insoluble matrix proteins secreted by cells grown under hypoxic conditions on hESCs, human mesenchymal stem cells (hMSCs), fibroblasts, and a variety of human cancer cell lines.

Malignant cell lines that were seen to be inhibited by the ECM using the MTT assay *in vitro* were then combined with soluble or insoluble ECM material and tested in the tumor choriointic membrane (tumcam) model as well as being injected subcutaneously in mice. Tumor load was assessed in the CAM assay after 7 days and in the mice after 14 days. In addition, a mouse model for human carcinomatosis was established by intraperitoneal injection of the human cell line labeled with green fluorescent proteins into nude mice. Once tumor load was evident, mice received weekly injections of soluble ECM.

In order to understand the mechanism behind effects on the tumors histologies were examined and *in vitro*

Fig. 1. Hypoxic dermal fibroblasts express stem cell-associated proteins. Hypoxic HDFs stained for stem cell-associated proteins (green) and counterstained with phalloidin (red) and DAPI (blue) to demonstrate the cytoskeleton (actin) and nuclei, respectively. (A) Nodal; (B) Brachyury; (C) Nestin; (D) Oct4 (HRP detection system) (Mag. = 40×).

staining was performed to look for any signs of apoptosis.

Results

Hypoxic conditions of 1~5% oxygen resulted in the cells differentially expressing over 5000 genes as compared to cells grown in otherwise identical but normoxic environments. Several of the upregulated genes expressed in the hypoxic cells are associated with pluripotent and follicular stem cells including Lnx2, SOX21, Nestin, NFATc1, Krt15, POU5F1 (OCT4), SOX2 and Nanog. In addition to gene upregulation, stem cell markers are se-

Gene	Fold Increase
LAMG3	3.99
LAMA5	3.37
LAMG2	2.92
LAMA1	2.01
LAMB1	21.45
LAMG1	19.95
LAMA4	17.77
LAMA2	9.58
COL4A5	17.86
CXCL12	47.8
NID2	8.87
NID1	4.45
NOTCH2	15.7

Fig. 2. Hypoxia stimulates a hECM associated with stem cell niches and anti-tumor activity.

Fig. 3. hESCs proliferate on hECM in cell culture for over 12 weeks.

creted, including Nestin, Oct 4, Brachyury, and Nodal (Fig. 1). A number of growth factors and morphogens such as VEGF and follistatin are upregulated and secreted in significant quantities as compared to normoxic cultures. Extracellular matrix proteins upregulated by hypoxia include many of which have been shown to be associated with stem cell niches, including stem cell niches in the skin, bone marrow, and spinal cord (Fig. 2). Several up-regulated compounds have been associated with anti-cancer activity (10). Some of the developmental proteins in hECM include lumican, fibromodulin and collagen XII. The hECM has characteristics identical to those that have been shown to support the growth of ES and other stem cells. The ECM supported the growth of hESCs for over 12 weeks in culture (Fig. 3) as well as the growth of human MSCs (Fig. 4).

A dose-dependant reduction in melanoma cells was seen *in vitro* with the soluble ECM and the addition of the soluble or insoluble extracellular matrix components secreted by the hypoxia-induced multipotent cells significantly reduced tumor load with human melanoma in the tumcam assay and when injected subcutaneously into the nude

Fig. 4. hECM improves hMSC monolayer culture expansion.

mouse model (Fig. 5).

Histological analysis showed that cancer cells exposed to ECM were condensed and appeared to be undergoing a controlled cell death. *In vitro* testing showed that the ECM upregulated Caspase 9 and induced apoptosis (Fig. 6). This result is consistent with that reported with follistatin (11) which is found in large quantities in the media produced by the multipotent cells grown under hypoxia. This result was seen in both rapidly dividing cancer cell lines as well as cancer stem cells. Normal stem cells and mature non-malignant cells grew well on the ECM. In addition to melanoma, research with ECM has demonstrated its ability to diminish or eliminate tumor load in adenocarcinoma (MDA-MB-435), colon cancer (HT29) and glioma (C6) in both *in vitro* and *in vivo* animal studies. Early work with the carcinomatosis model showed that weekly intraperitoneal injections of ECM significantly reduced tumor load within three weeks of the initial treatment. Longer term studies to assess treatments to eliminate all signs of tumor are underway.

Discussion

Research has shown the reversal of the developmental program of adult cells through a variety of means, including the exposure of cells to pluripotent cell extracts (12, 13), and the viral transduction of stem cell genes encoding for transcription factors (14-16). Recent research has shown the ability to induce stem cell gene expression in adult human fibroblasts without the use of genetic manipulation (17). We have shown that mimicking the embryonic environment with hypoxia and suspension culture results in the upregulation of genes that are associated with pluripotent and follicular stem cells including LnX2, SOX21, Nestin, NFATc1, Krt15, POU5F1 (OCT4), SOX2 and Nanog. In addition to gene upregulation, stem cell markers are secreted, including Nestin, Oct 4, Brachyury, and Nodal, which indicated that the embryonic conditions induce the fibroblasts to become multipotent. Conditioned media from these hypoxic cultures has been shown to support MSCs and ESCs *in vitro* as well as the growth of tri-

Fig. 5. hECM oncological applications - reduction of melanoma tumor size in mouse and fertilized chick egg assay (* $p < 0.05$).

Fig. 6. ECM induces Caspase 9 and cell apoptosis in a dose dependent manner.

chogenic cells (18). In addition, injection of the soluble proteins produced by the hypoxic cultures has induced a statistically significant increase in hair growth in patients with male pattern baldness (19). This result persisted over one year, even though the injection was at baseline only, supporting the hypothesis that the soluble ECM stimulated stem cells to produce more hairs in each follicle as well as new follicle formation. The soluble ECM contains large quantities of follistatin, laminins, and decorins, each of which has been shown to induce malignant cells to move down a more normal phenotype. We have shown that both soluble and insoluble forms of our embryonic-like ECM induces the upregulation of Caspase 9 which forces a variety of cancer cell lines and cancer stem cells into apoptosis. These results support the further study with the embryonic ECM to induce stem cells in muscle and other tissue to regenerate as well to reduce tumor mass of inoperable solid tumors and treat cancers which do not respond to currently available treatments.

Potential conflict of interest

The following authors disclose potential conflict of interest as current employees of Histogen: Emmett Pinney, Michael Zimmer, Mayra Montes-Camacho, Gail K. Naughton.

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