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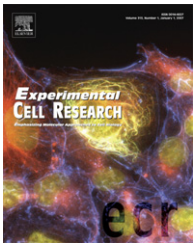

Research Article

# Glioma-associated endothelial cells show evidence of replicative senescence

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ABSTRACT

The innately programmed process of replicative senescence has been studied extensively with respect to cancer, but primarily from the perspective of tumor cells overcoming this stringent innate barrier and acquiring the capacity for unlimited proliferation. In this study, we focus on the potential role of replicative senescence affecting the non-transformed endothelial cells of the blood vessels within the tumor microenvironment. Based on the well-documented aberrant structural and functional features of blood vessels within solid tumors, we hypothesized that tumor-derived factors may lead to premature replicative senescence in tumor-associated brain endothelial cells (TuBEC). We show here that glioma tissue, but not normal brain tissue, contains cells that express the signature of replicative senescence, senescence-associated β-galactosidase (SA-β-gal), on CD31-positive endothelial cells. Primary cultures of human TuBEC stain for SA-β-gal and exhibit characteristics of replicative senescence, including increased levels of the cell cycle inhibitors p21 and p27, increased resistance to cytotoxic drugs, increased growth factor production, and inability to proliferate. These data provide the first demonstration that tumor-derived brain endothelial cells may have reached an end-stage of differentiation known as replicative senescence and underscore the need for anti-angiogenic therapies to target this unique tumor-associated endothelial cell population.



## Introduction

The growth of solid tumors has been correlated with access to a blood supply [1]. A source of these vessels is the surrounding vasculature that is activated by proangiogenic cytokines and growth factors, particularly VEGF and IL-8, which are produced by the tumors themselves [2–4]. This tumor microenvironment destabilizes existing blood vessels, induces endothelial cells to migrate and proliferate, and leads to new vessel formation within the tumor [2–5]. Indeed, blood vessels in the

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periphery of tumors, particularly in gliomas, exhibit highly proliferating endothelial cells. A histological examination of the vasculature within the tumor reveals that these blood vessels are structurally and functionally very different from normal vessels [6,7]. Tumor-associated blood vessels are leaky and hemorrhagic, displaying tortuous and disorganized structures, while normal vessels form orderly networks of vessels with tight junctions [6,7]. It has been proposed that the tumor microenvironment stimulates the development of this abnormal vasculature; however the stimuli for the induction of these aberrant vessels within the tumor have not been confirmed [6]. It is known that the tumor environment produces growth factors which stimulate endothelial cell proliferation (e.g., VEGF), and this tumor environment is often hypoxic and usually depleted of essential nutrients [4]. Blood vessels within the tumor are therefore constantly exposed to a tumor microenvironment, which is highly abnormal, for extended periods of time, perhaps months or even years. The tumor microenvironment, containing secreted growth factors, will constantly stimulate the normal endothelial cells to proliferate to form new vessels. This is in sharp contrast to endothelial cells in the normal brain environment, which undergo very few cell divisions and are considered a relatively static population [8].

The data presented here provide the first demonstration that the effect of the proangiogenic tumor environment on the tumor-associated brain endothelial cells (TuBEC) is to induce these cells to reach an end-stage of differentiation known as replicative senescence. The hypothesis of this study is that the abnormal appearance and behavior of TuBEC are due to an underlying functional change, the induction of the senescent-like phenotype. The studies presented here test and confirm this hypothesis. Senescence is an irreversible end-stage which can be induced by excessive proliferation as well as other stimuli, such as oxidative stress [8–11]. Studies have shown that the serial exposure of endothelial cells to VEGF accelerates accumulation of senescent cells [10]. Replicative senescent cells are highly active, expressing unique characteristics and functions. These cells are noted for an irreversible arrest in G1 phase of the cell cycle, displaying increased levels of the cdk inhibitors p21, p27, and p16, increased expression of p53, cyclin D1, and cyclin E, and a decreased expression of cyclin A [12–16]. Replicative senescent cells are generally resistant to apoptosis, but this is cell type dependent (e.g., T cells or fibroblasts) and species dependent [8,17,18]. Senescent fibroblasts were shown to be metabolically active, secrete growth factors and cytokines, and thereby stimulate tumor growth [8,17]. Replicative senescent endothelial cells exhibit unique morphological changes including an increase in cell size and nuclear size and the development of a flat, veil-like appearance [14]. Furthermore, replicative senescent cells were shown to stain for β-galactosidase, a recognized marker for senescent cells [9]. Senescence-associated β-galactosidase (SA-β-gal) staining has been correlated with the morphological changes observed in replicative senescent cells [14,18,19].

In an earlier report, we demonstrated that TuBEC have unique morphologic and functional features compared to primary cultures of normal brain-derived endothelial cells (BEC) [7]. TuBEC are large, irregularly shaped, and demonstrate a veil-like appearance, unlike normal endothelial cells. Furthermore, TuBEC cultures exhibit a markedly reduced rate of proliferation and a decreased rate of apoptosis as compared to BEC [7]. These unique features of TuBEC were reminiscent of replicative senescent cells [8,20,21]. In this study we analyzed the functional properties of TuBEC. Our results show that tumor-associated endothelial cells stain positively for SA-β-gal both in tumor tissue and in culture. Furthermore, TuBEC are arrested at the $G_1$ phase of the cell cycle, are more resistant to cytotoxic drugs, and produce more growth factors compared to normal BEC. These data support the notion that TuBEC have the characteristics of replicative senescent cells.

## Methods

### *Cell culture*

TuBEC and BEC were isolated from glioma and normal human brain tissue respectively as previously described previously [7]. All tissues were obtained within the Keck School of Medicine, University of Southern California Institutional Review Board guidelines. Diagnostic information pertaining to the tissues used in this study is summarized in Table 1. The average ages of tumor patients (38.4±3.8) and control specimens (37.3±3.2) were similar. Endothelial cells were cultured in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 100 ng/ml endothelial cell growth supplement (ECGS) (Upstate Biotechnologies, Rochester, NY), 2 mM L-glutamine (GIBCO), 10 mM HEPES (GIBCO), 24 mM sodium bicarbonate (GIBCO), 300 U heparin USP (Sigma-Aldrich, St. Louis, MO), 1% penicillin/streptomycin (GIBCO), and 10% fetal calf serum (FCS) (Omega Scientific, Tarzana, CA). A172 and LN229 glioblastoma cell lines were cultured in DMEM and RPMI 1640 (GIBCO) media, respectively, supplemented with 10% FCS. Purity of endothelial cell cultures was analyzed at each passage by immunostaining with the following specific endothelial cell markers: CD31 (PECAM-1), von Willebrand factor (vWF), and CD105 (endoglin); cells were routinely greater than 98% positive for these markers. The endothelial cell characteristic

**Table 1 – Information of TuBEC and BEC patients**

| Cell | Diagnosis | Age | Sex | Treatment |
|---|---|---|---|---|
| TuBEC 1 | Anaplastic astrocytoma | 45 | F | Untreated |
| TuBEC 2 | Glioblastoma multiforme | 26 | M | Treated (radiation) |
| TuBEC 3 | Glioblastoma multiforme | 46 | F | Treated (radiation) |
| TuBEC 4 | Oligodendroglioma | 41 | M | Untreated |
| TuBEC 5 | Glioblastoma multiforme | 34 | M | Untreated |
| BEC 1 | Trauma | 49 | F | Untreated |
| BEC 2 | Trauma | 42 | F | Untreated |
| BEC 3 | Trauma | 33 | M | Untreated |
| BEC 4 | Epilepsy | 40 | F | Untreated |
| BEC 5 | Trauma | 32 | M | Untreated |
| BEC 6 | Trauma | 28 | F | Untreated |

Brain tumor tissues were obtained from glioma patients; control brain specimens were primarily obtained from trauma patients.