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ScienceDirect
Toxicology in Vitro 21 (2007) 1619–1630
Toxicology in Vitro
www.elsevier.com/locate/toxinvit

An *in vitro* embryotoxicity assay based on the disturbance of the differentiation of murine embryonic stem cells into endothelial cells. I: Establishment of the differentiation protocol

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Received 16 February 2007; accepted 27 June 2007
Available online 21 July 2007

Abstract

The aim of the present study was to establish an experimental protocol to differentiate murine embryonic stem (ES) cells into endothelial cells *in vitro*. The spinner flask technique as well as the hanging drop method were used to generate so-called embryoid bodies (EBs). In order to find out the optimal differentiation environment, EBs were cultured under various experimental conditions for up to 14 days. The influence of basic fibroblast growth factor (bFGF) alone, vascular endothelial growth factor (VEGF) alone, bFGF and VEGF together and a cocktail consisting of bFGF, VEGF, interleukin-6 (IL-6) and erythropoietin (Epo) on the induction of differentiation of ES cells into endothelial cells was studied. Different concentrations of growth factors and times of treatment were applied. Endothelial cells were characterized by analyzing the expression of platelet-endothelial cell adhesion molecule (PECAM-1), the endothelial-specific vascular endothelial cadherin (VE-Cadherin), the angiopoietin receptor Tie-2, VEGF receptors 1 and 2 (Flt-1 and Flk-1, respectively) and the soluble form of Flt-1 (sFlt) at the mRNA level. PECAM-1 and VE-Cadherin were also studied at the protein level. The data clearly showed that EBs generated by the hanging drop method, followed by their transfer into suspension culture on day 3 of differentiation and their subsequent plating on day 5 is the best of the studied methods to differentiate ES cells into endothelial cells. Addition of VEGF alone or a cocktail consisting of VEGF, bFGF, IL-6 and Epo resulted in the strongest gene expression levels of the above mentioned endothelial cell markers in the differentiated ES cells.

Keywords: Murine; Embryonic stem cells; Differentiation; Embryoid body; Endothelial cells; bFGF; VEGF; VE-Cadherin; PECAM-1

1. Introduction

Murine embryonic stem (ES) cells are pluripotent cells derived form the inner cell mass of the blastocyst. They can be kept in their undifferentiated status when cultured in the presence of the leukaemia inhibitory factor (LIF) (Williams et al., 1988). As soon as LIF is removed ES cells start to differentiate into cells of all three germ layers, such as cardiac muscle cells, neuronal cells or endothelial cells (Doetschman et al., 1985).

In the developing embryo the differentiation of mesodermal cells into endothelial cells and the formation of a complex, mature tubular network is a multifaceted process,

Abbreviations: aFGF, acidic fibroblast growth factor; ANG, angiopoietin; bFGF, basic fibroblast growth factor; EB, embryoid body; Epo, erythropoietin; ES cells, embryonic stem cells; Flk-1, fetal liver kinase (VEGF receptor 2); Flt-1, Fms-like tyrosine kinase (VEGF receptor 1); GF-cocktail, growth factor cocktail; IL-6, interleukin-6; LIF, leukaemia inhibitory factor; PECAM-1, platelet-endothelial cell adhesion molecule-1; sFlt, soluble Flt-1; Tie-2, tunica interna endothelial cell kinase 2 (angiopoietin receptor); VE-Cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor.

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doi:10.1016/j.tiv.2007.06.018

which can be subdivided into vasculogenesis and angiogenesis. Vasculogenesis is the process, in which angioblasts differentiate from the mesoderm and assemble to primitive blood vessels (Risau et al., 1988). Angiogenesis, however, is defined as the process of growth, expansion and remodelling of these primitive vessels into a mature tubular network and involves the sprouting of new blood vessels from pre-existing ones as well as the longitudinal division of existing ones (Risau et al., 1988; Conway et al., 2001).

Both processes strongly depend on a balance between positive and negative regulatory factors (Risau and Flamme, 1995; Hanahan, 1997; Risau, 1997; Carmeliet and Collen, 1999; Yancopoulos et al., 2000). The vascular endothelial growth factor (VEGF) is a mitogen selective for vascular endothelial cells *in vitro* and *in vivo*. Furthermore, VEGF signaling is essential for the generation of blood vessels but not for their maintenance (Hirashima et al., 1999; Argraves et al., 2002). It is already expressed at early stages of embryogenesis (around day 7.5) by cells of endodermal origin (Breier et al., 1995).

The notion that VEGF is an important regulator of vasculogenesis is supported by the fact that the VEGF receptor 2 (Flk-1 in mouse) is the first marker being expressed in a mesodermal cell population giving rise to endothelial cell precursors (Hirashima et al., 1999). In mouse embryos Flk-1 is detected from day 7 onwards (Yamaguchi et al., 1993). Flk-1 as well as the VEGF receptor 1 (Flt-1) belong to the tyrosine kinase receptors with seven Ig-like domains in the extracellular domain, a transmembrane domain, and a tyrosine kinase domain. Flt-1 shows a 10-fold higher affinity to VEGF even as the truncated soluble splice variant of Flt-1 (sFlt), but a 10-times lower kinase activity than Flk-1 (Waltenberger et al., 1994; Breier et al., 1995). Both receptors are co-expressed on endothelial cells and were shown to be very important regulators of endothelial cell differentiation and maturation (Breier et al., 1995). While Flk-1 was shown to be required for endothelial cell differentiation and proliferation, Flt-1 acts as a negative regulator of endothelial cell proliferation (Shalaby et al., 1997; Kearney et al., 2002). sFlt lacks the membrane-proximal seventh Ig-like domain, binds VEGF with high affinity and efficiently inhibits VEGF-induced mitogenesis of vascular endothelial cells in culture (Kendall and Thomas, 1993).

Cytokines such as interleukin-6 (IL-6) and erythropoietin (Epo) were thought to be specific for the hematopoietic system. However, IL-6 and Epo are also known to affect several functions of endothelial cells. The Epo receptor is expressed on human endothelial cells *in vitro* and *in vivo* (Anagnostou et al., 1994). Epo stimulates angiogenesis and endothelial sprouting (Ribatti et al., 1999). IL-6 also plays a physiological role in angiogenesis (Cohen et al., 1996) and stimulates the motility of endothelial cells (Rosen et al., 1991).

The fibroblast growth factors (FGFs), especially the acidic and the basic FGF (aFGF and bFGF, respectively), exhibit angiogenic activity and are chemotactic for endothelial cells (Folkman and Klagsbrun, 1987; Flamme and Risau, 1992; Doetschman et al., 1993; Gendron et al., 1996; Bikfalvi et al., 1997). The bFGF receptor is expressed on undifferentiated human ES cells (Schuldiner et al., 2000) as well as on endothelial precursor cells (Burger et al., 2002). Endothelial cell migration and division, proteolytic degradation of vessels, as well as capillary tube formation are effects caused by FGFs (Slavin, 1995). However, bFGF was also shown to inhibit kidney vessel development (Kloth et al., 1998), thus indicating that bFGF has a morphogenic rather than a mitogenic function. bFGF as well as VEGF can prevent endothelial cells from undergoing apoptosis (Korff and Augustin, 1998).

Endothelial cells are linked to each other by tight and adherens-type junctions. An adherens-type junction protein strictly specific for endothelial cells is the vascular endothelial cadherin (VE-Cadherin). Widely expressed as early as from day 7.5 onwards, its distribution along the vascular tree suggests an important role during the process of vessel organisation (Breier et al., 1996; Dejana, 1996; Gory-Faure et al., 1999). The platelet-endothelial cell adhesion molecule-1 (PECAM-1) is a transmembrane protein abundantly expressed early in vascular development on endothelial cells, but also on monocytes, circulating platelets and neutrophils (Albelda et al., 1990; Newman, 1997). Angiopoietins (ANGs) are angiogenic factors, which act through binding to their receptor, the tunica interna endothelial cell kinase 2 (Tie-2). At early stages of angiogenesis ANGs regulate the stabilisation of primitive vessels (Davis et al., 1996; Maisonpierre et al., 1997).

A number of *in vitro* protocols for the differentiation of ES cells into endothelial cells have been developed. In this context *in vitro* endothelial cell differentiation and blood vessel formation have been observed in so-called embryoid bodies (EBs). EBs are three-dimensional cell clusters, in which ES cells differentiate into cells with gene expression patterns resembling the developmental pattern during embryogenesis (Guan et al., 1999). Endothelial cell differentiation in EBs has been shown to occur by applying the suspension culture technique (Doetschman et al., 1985; Risau et al., 1988; Doetschman et al., 1993; Yamaguchi et al., 1993), the culture in a semisolid medium (Vittet et al., 1996; Robertson et al., 2000), the culture in or on Matrigel (Doetschman et al., 1993), the hanging drop method (Goumans et al., 1999) or the spinner flask technique (Wartenberg et al., 1998).

The aim of this study was to establish a simple and fast differentiation protocol of ES cells into endothelial cells, which could be used as an *in vitro* assay to screen for the embryotoxic potential of compounds. In the accompanying paper the embryotoxic potential of six test compounds was investigated by analyzing their capacity to inhibit the differentiation of ES cells into endothelial cells.

In the first part of this report different cell culture techniques were applied in order to differentiate ES cells under optimal experimental conditions. The spinner flask technique (Wartenberg et al., 1998) as well as the hanging drop method (Wobus et al., 1991) were used to generate EBs.