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Vitronectin supports migratory responses of corneal epithelial cells to substrate bound IGF-I and HGF, and facilitates serum-free cultivation

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Abstract

Vitronectin (VN) is a multi-functional glycoprotein best known for its effects on cell attachment and spreading, but has more recently been shown to mediate cellular responses to growth factors. The presence of VN within the tear film and expression of required receptors (alpha v integrins) on corneal epithelial cells suggests the potential for a similar role within the ocular surface. Thus we have studied the ability of VN to alter the metabolic (MTT assay) and migratory (trans-membrane migration) responses of corneal epithelial cells to growth factors associated with the ocular surface including epidermal growth factor (EGF), hepatocyte growth factor (HGF), keratinocyte growth factor (KGF) and insulin-like growth factor-I (IGF-I). Our hypothesis was that culture surfaces coated with VN might selectively facilitate responses to growth factors which are known to bind VN including EGF, IGF-I (via IGF binding protein) and HGF. Metabolic responses were observed towards each growth factor when applied to the culture medium, but not towards culture plastic pre-treated with VN and, or growth factors. Optimal metabolic responses were observed towards IGF-I applied in conjunction with EGF. Migration through porous polycarbonate membrane was significantly increased when the substrate had been pre-coated with VN and IGF-I (applied in conjunction with IGFBP-3) or VN and HGF. This finding is consistent with the ability of IGF-I (via an IGFBP) and HGF to form complexes with VN and suggests that integrin/growth factor receptor co-activation is required for corneal epithelial cell migration. In further studies, VN applied in conjunction with IGF-I, IGFBP-3 and EGF (both to the culture plastic and in the culture medium) was found to support the establishment and serial propagation of limbal-corneal epithelial cell cultures in the absence of serum, but irradiated 3T3 cells (i3T3) were still necessary for culture expansion. Immunocytochemistry of resulting cultures for keratin 3 and p63 revealed a similar phenotype to those established under current best-practice conditions (i3T3, foetal bovine serum, EGF and insulin). In conclusion, our novel findings suggest a role for VN-growth factor complexes in stimulating corneal epithelial migration within the provisional wound bed and demonstrate that VN-growth factors interactions can be exploited to enable manufacture of bioengineered ocular surface tissue under serum-free conditions.

Keywords: cornea; limbus; epithelium; vitronectin; IGF-I; HGF; migration; serum-free culture

1. Introduction

Vitronectin (VN) is a 75 kDa glycoprotein found predominantly in blood and is best known for its ability to promote attachment and spreading of cells *in vitro* (Preissner, 1991; Schvartz et al., 1999). Cellular responses to VN are mediated via α_v integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) which recognise an Arg-Gly-Asp (RGD) sequence adjacent to the protein's N-terminus (Pytela et al., 1985). Extravascular deposits of VN *in vivo* support the attachment of cells involved with wound repair and promote angiogenesis (Nisato et al., 2003). Additional VN functions are mediated via binding to proteins involved with immune defence, coagulation, fibrinolysis and the extracellular matrix (ECM) (Schvartz et al., 1999), but the present study extends from the discovery of interactions between VN

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and growth factors (Clemmons and Maile, 2005; Upton et al., 1999).

It has been suggested that VN bound within the provisional wound bed may provide a growth factor delivery system (Upton et al., 1999). This theory is supported through the demonstration of VN binding to growth factors including epidermal growth factor (EGF) and fibroblast growth factor (Schoppet et al., 2002), hepatocyte growth factor/scatter factor (HGF/SF) (Rahman et al., 2005), insulin-like growth factor-II (IGF-II) (Upton et al., 1999) and IGF-I (via IGF-binding proteins or IGFBPs) (Kricker et al., 2003). The functional significance of these interactions has been confirmed through observation of cellular responses to culture plastic pre-treated with VN and growth factors (Nam et al., 2002; Noble et al., 2003). We have recently optimised these VN-growth factor combinations for the cultivation of skin keratinocytes (Dawson et al., 2006; Hollier et al., 2005; Hyde et al., 2004). The similarities in growth factor requirements observed between skin keratinocytes and corneal epithelial cells (Germain et al., 2000; Pellegrini et al., 1997) has led us presently to consider the potential significance of VN-growth factor interactions to the ocular surface.

VN is present in tears (0.6 μg/ml) and is elevated ten-fold following periods of eye-lid closure (Sack et al., 1994). The majority of VN originates from conjunctival blood vessels (Sack et al., 1994), but VN has also been reported within the basement membrane of the corneal epithelium suggesting a possible endogenous source (Xiao et al., 2005). Competency to respond is demonstrated through expression of the $\alpha_v\beta_5$. VN-receptor on the surface of normal corneal epithelial *in situ* (Rayner et al., 1998). It is thus possible that tear film VN acting alone or in conjunction with locally produced growth factors and/or ECM proteins may contribute to maintenance of a normal ocular surface. Indeed, topically applied VN (0.2 mg/ml) promotes healing of the corneal epithelium in a rabbit model (Kabata et al., 1990). It is also possible that the presence of VN in serum (0.3 mg/ml) (Hogasen et al., 1993) may contribute to the efficacy of autologous serum eye drops as utilised in the treatment of persistent epithelial defects and dry eye (Geerling et al., 2004).

The aim of the present study was therefore to examine the effects of VN on cellular responses to growth factors associated with corneal re-epithelialisation. We have utilised cultures derived from donor limbal epithelium since this tissue regenerates the corneal epithelium *in situ* (Davanger and Evensen, 1971; Schermer et al., 1986) and provides a source of epithelial progenitor cells for tissue engineering applications (Pellegrini et al., 1997; Schwab, 1999). Growth factors examined include IGF-I (in conjunction with IGFBP-3), EGF, HGF and keratinocyte growth factor (KGF), based upon prior demonstration of their interactions with VN and/or a significant role in corneal re-epithelialisation (Imanishi et al., 2000; Klenkler and Sheardown, 2004; Wilson et al., 1999a). Measures of cellular response include the MTT assay of metabolic activity, transmembrane migration and serial cultivation in the presence of murine 3T3 feeder cells. The phenotype of resulting cultures has also been examined via immunocytochemistry for p63 and keratin 3.

2. Materials and methods

2.1. Routine establishment of limbal-corneal epithelial cultures

Human limbal tissue was obtained with donor and institutional ethics committee consent, and processed as previously to isolate limbal epithelial cells (Barnard et al., 2001). Cultures were established in the presence of gamma irradiated (50 Gy) murine 3T3 cells (ATCC; CCL-92) using a 3:1 mixture of DMEM (Invitrogen, 10313021) and Ham's F12 medium (Invitrogen; 11765-054) supplemented with 10% foetal bovine serum (FBS; Thermo Trace, Melbourne, Australia; 15-010-0500V), 1 μg/ml insulin (Sigma-Aldrich; I6634), 10 ng/ml human recombinant EGF (Invitrogen; 13247-051), 180 μM adenine (Sigma-Aldrich; A9795), 0.1 μg/ml cholera toxin (Sigma-Aldrich; C8052), 2 mM L-glutamine (Invitrogen; 25030149), 0.4 μg/ml hydrocortisone (Sigma-Aldrich; H4001), 0.01% v/v non-essential amino acids solution (Invitrogen; 11140-050), and 1% v/v penicillin/streptomycin solution (Invitrogen; 15070-063). This same serum supplemented medium was used as a positive control throughout comparisons with VN/growth factor treatments and is subsequently referred to by the abbreviation SSM.

2.2. MTT assay

The MTT assay measures conversion of methyl-thiazolyldiphenyl-tetrazolium bromide (MTT) by mitochondrial enzymes (Ealey et al., 1988) and was performed in 24-well (1.8 cm^2) culture plates (Nunc; 142475) using cells from early passage cultures established using the standard methods outlined above. Cells were seeded at a density of between 3 to 5×10^4 cells per well and cultured for 72 h. These assays were performed in the absence of 3T3 feeder cells. Following exposure to varying formulations of VN and growth factors each culture was incubated in 0.5 mg/ml MTT solution (Sigma-Aldrich; M5655) for 2 h at 37 °C with the converted product being extracted with acidified isopropyl alcohol and measured by absorbance at 550 nm minus 650 nm.

Vitronectin (VN) affinity purified from human plasma (Promega, G538A) was applied as 300 ng in 300 μl of DMEM to each well (1.8 cm^2) and allowed to absorb to the culture plastic during overnight incubation at 4 °C. The excess VN (unbound fraction) was removed prior to addition of growth factors or cells. Growth factors were subsequently applied diluted in DMEM and incubated for 3 h at 37 °C. During initial studies of responses to IGF-I, IGFBP-3 and EGF (Fig. 1), responses were examined under two parallel conditions: (1) when the growth factor solutions used to pre-treat the culture plastic were retained during the 72-h culture; and (2) when the growth factor solutions were replaced with serum-free growth factor free medium prior to seeding the cells. For subsequent studies involving HGF and KGF, only the first treatment condition was examined (growth-factor solution retained in presence of cells). The amounts of IGF-I (100 ng/well; GroPep, Adelaide, SA, Australia), non-glycosylated IGFBP-3 (500 ng/well; N109D analogue, Auspep, Parkville,