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Granzyme B cleavage of fibronectin disrupts endothelial cell adhesion, migration and capillary tube formation

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ABSTRACT

Dysregulated angiogenesis contributes to the pathogenesis of chronic inflammatory diseases. Modulation of the extracellular matrix by immune-derived proteases can alter endothelial cell–matrix interactions as well as endothelial cell sprouting, migration and capillary formation. Granzyme B is a serine protease that is expressed by a variety of immune cells, and accumulates in the extracellular milieu in many chronic inflammatory disorders that are associated with dysregulated angiogenesis. Although granzyme B is known to cleave fibronectin, an essential glycoprotein in vascular morphogenesis, the role of granzyme B in modulating angiogenesis is unknown. In the present study, granzyme B cleaved both plasma fibronectin and cell-derived fibronectin, resulting in the release of multiple fibronectin fragments. Granzyme B cleavage of fibronectin resulted in a dose-dependent reduction in endothelial cell adhesion to fibronectin as well as reduced endothelial cell migration and tubular formation. These events were prevented when granzyme B activity was inhibited by a small molecule inhibitor. In summary, granzyme B-mediated cleavage of fibronectin contributes to attenuated angiogenesis through the disruption of endothelial cell — fibronectin interaction resulting in impaired endothelial cell migration and tubular formation.

1. Introduction

Angiogenesis, the formation of new capillaries from pre-existing vessels, is a tightly regulated process that is critical for normal development, ensuring adequate blood supply to the target tissue (Carmeliet, 2000). However, dysregulated angiogenesis, which refers to the formation of unstable, incomplete and immature neovessels, plays a pivotal role in several pathological conditions including cancer and other chronic inflammatory diseases (Carmeliet, 2003; Szekanecz and Koch, 2007). The formation of immature, unstable capillaries promotes inflammation by enabling immune cell infiltration across the endothelium. Conversely, formation of a stable capillary network is required for adequate resolution of inflammation and initiation of repair processes (Jackson et al., 1997). As such, properly orchestrated angiogenesis during inflammation is considered a key determinant for the progression of numerous chronic inflammatory disorders (Costa et al., 2007).

Granzyme B (GZMB) is a serine protease that is expressed and released by a variety of immune cells and accumulates in the extracellular matrix (ECM) during chronic inflammatory diseases that are highly associated with dysregulated angiogenesis (Boivin et al., 2009; Hendel et al., 2010). The intracellular delivery of GZMB, a process that requires the release of this enzyme together with the pore forming molecule, perforin, constitutes the central mechanism in the granule exocytosis pathway by which cytotoxic immune cells mediate target cell apoptosis in order to eliminate viral infected and/or transformed cells (Waterhouse et al., 2004). However, work from our laboratory and others suggests additional extracellular roles for GZMB as several ECM proteins are cleaved by GZMB (Boivin et al., 2009). Moreover, under certain conditions, GZMB is expressed and released by other immune cells such as neutrophils, dendritic cells, macrophages, basophils and mast cells (Hendel et al., 2010) as well as non-immune cells such as chondrocytes and keratinocytes (Hernandez-Pigeon et al., 2006, 2007; Saito et al., 2008). These findings highlight the extracellular role of GZMB in inflammation that can occur independently of target cell recognition and/or entry, and involves non-directional GZMB release. Indeed, GZMB is constitutively released from stimulated natural killer (NK) cells and cytotoxic T lymphocytes (CTL) in the absence of target cell engagement (Isaaz et al., 1995; Prakash et al., 2009). While emerging evidence suggests that extracellular GZMB activity can impair tissue integrity, the direct consequences of GZMB-mediated ECM cleavage on the angiogenic process have yet to be explored.

The interaction of endothelial cells (EC) with the surrounding ECM, in concert with tightly regulated pericellular proteolysis, is critical for neovessel formation (Davis et al., 2002; Sottile, 2004; van Hinsbergh et al., 2006). Initial proteolysis of basal lamina proteins and controlled breakdown of surrounding ECM are required to liberate EC, allowing

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sprouting into the interstitium (Carmeliet, 2000; Davis and Senger, 2005). Subsequent interaction between ECM proteins that contain cell binding domains, namely RGD (Arg-Gly-Asp) domains, with integrin receptors on EC cell surface facilitate EC adhesion, migration and support correct alignment for EC to form functional capillaries (Hynes, 2007).

Fibronectin (FN) is an important ECM protein that has a central role in regulating vascular morphogenesis (Astrof and Hynes, 2009). FN is a large glycoprotein consisting of two similar but not identical ~250 kDa monomers linked by a disulfide bond at the C-termini. This protein possesses multiple binding domains that bind ECM proteins, cell surface integrins and growth factors (Pankov and Yamada, 2002). The central cell binding domain, consisting of the RGD motif, is essential for binding $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins that are required for EC adhesion and migration (Astrof and Hynes, 2009). FN circulates at high levels in the plasma in a closed globular structure, and is produced by EC, smooth muscle cells (SMC) and fibroblasts (Mosher, 1989). During inflammation, FN accumulates in the affected area due to leakage from capillaries. Once incorporated in the ECM, FN undergoes a conformational change, exposing matricryptic RGD binding domains that facilitate cell adhesion and migration as part of the provisional matrix formation during inflammation (Vartio et al., 1987; Mosher, 1989). Further alteration of FN biological activities are attributed to proteolytic processing by ECM proteases (Wayner et al., 1988). Thus, FN proteolysis during inflammation modulates EC-matrix interactions and angiogenesis (Arroyo and Iruela-Arispe, 2010).

Previous studies suggest that GZMB cleaves FN at the RGD binding domain, implying that GZMB-mediated FN proteolysis may alter cell–matrix interactions (Buzza et al., 2005). Indeed GZMB induces detachment and anoikis in EC, SMC and fibroblasts (Choy et al., 2004a; Buzza et al., 2005; Pardo et al., 2007). However, during the angiogenic process, detachment of EC from a quiescent monolayer and destabilization of the basal lamina are required in order to initiate EC sprouting and migration (Carmeliet, 2000; Davis and Senger, 2005; Davis, 2011). Moreover, proteolytic alteration of the pericellular matrix may expose cryptic domains in FN that further support or inhibit EC angiogenesis (Davis et al., 2000; van Hinsbergh et al., 2006). Since immune-derived proteases are believed to regulate the angiogenic response during inflammation through ECM processing (Arroyo and Iruela-Arispe, 2010), it is important to explore the effects of GZMB-mediated FN cleavage on EC angiogenic behavior. In the current study we demonstrate that GZMB cleavage of FN impairs EC adhesion, migration and capillary tube formation.

2. Results

2.1. GZMB cleaves plasma and endogenous FN

To test whether GZMB cleaves plasma FN in its matrix form, plates were coated with human plasma FN and treated with increasing concentrations of GZMB. Western blot analysis of the supernatants identified a number of cleavage fragments that appear at greater intensity with increasing GZMB concentration, indicating that GZMB-mediated FN cleavage is dose-dependent (Fig. 1A). Pre-incubation of GZMB with Compound 20 (Willoughby et al., 2002) reduced the appearance of the FN cleavage fragments, confirming that GZMB is responsible for generating the observed FN fragments.

FN can be produced locally within the tissue by a large number of cells including SMC, fibroblasts and EC (Owens et al., 1986; Vartio et al., 1987; Glukhova et al., 1989). However, cells may produce a different

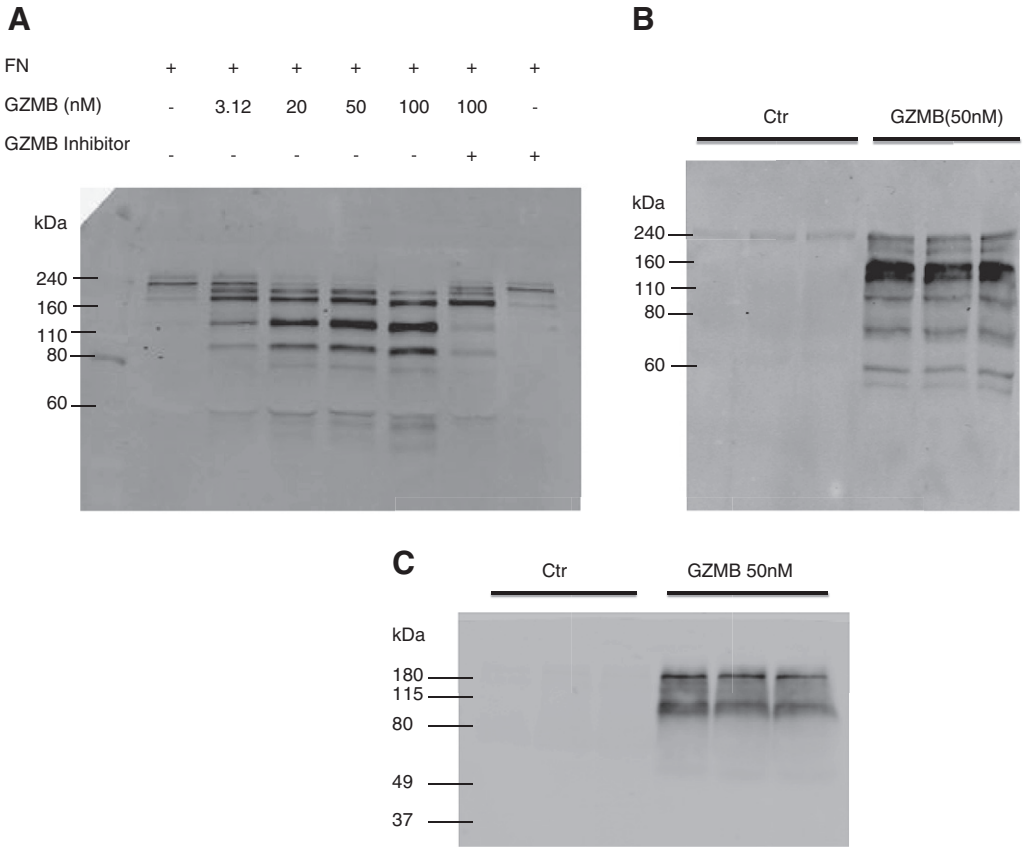

Fig. 1. GZMB cleaves plasma FN and cell-derived FN. A. Culture wells were coated with 20 μg/ml human plasma FN. Increasing concentrations of GZMB were added to FN coated wells, with or without GZMB inhibitor (Compound 20) and incubated for 24 h at RT. Supernatants were removed from the wells and FN fragments in the supernatants were detected by western blotting. B. HMVEC and C. Human SMC, were grown to confluence and maintained in serum-reduced media for 7 d. Cells were removed from the culture wells by adding NH_4OH followed by extensive washing. ECM was then treated with GZMB for 24 h at RT. Supernatants were removed from the wells and FN in the supernatants was detected by western blotting.