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Growth regulated oncogene is pivotal in thrombin-induced angiogenesis

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1. Background and summary

Although the association of thrombosis and cancer is well recognized, the role of thrombin in enhancing the malignant phenotype has only recently been appreciated. Thrombin enhances tumor implantation, migration, growth, metastasis and angiogenesis [1]. The focus of this review is on thrombin-induced angiogenesis. It has been shown that thrombin induces angiogenesis in the chick chorioallantoic model as well as endothelial cell tubule formation in a matrigel assay. Activation of thrombin-induced angiogenesis is preceded by and associated with the upregulation of various vascular growth factors and receptors which include VEGF and its KDR receptor, Angiopoietin-2 and metalloproteinase 1 and 2 (MMP-1 and -2). In this review, we discuss the role of the chemokine, Growth Regulated Oncogene alpha (GRO-α) in the upregulation of the same vascular growth factors cited. Thrombin markedly upregulates GRO-α in several tumor cell lines studied as well as primary endothelial cells (HUVEC and HBMEC). GRO-α is required for thrombin-induced upregulation of VEGF, KDR, Angiopoietin-2, MMP-1 and MMP-2. Knock down of GRO-α in B16F10 cells impairs thrombin upregulation of the cited vascular growth factors and receptor *in vitro* and tumor growth and angiogenesis *in vivo* in syngeneic mice. Thus GRO-α is pivotal in thrombin-induced angiogenesis.

2. Results

The mechanism of thrombin-induced angiogenesis is poorly understood. Using a gene chip array to investigate the pro-malignant phenotype of thrombin-stimulated cells we observed that thrombin markedly upregulates GRO-α in several tumor cell lines as well as endothelial cells by mRNA and protein analysis [2]. Thrombin enhanced the secretion of GRO-α from tumor cells 25–64 fold (Figure 1).

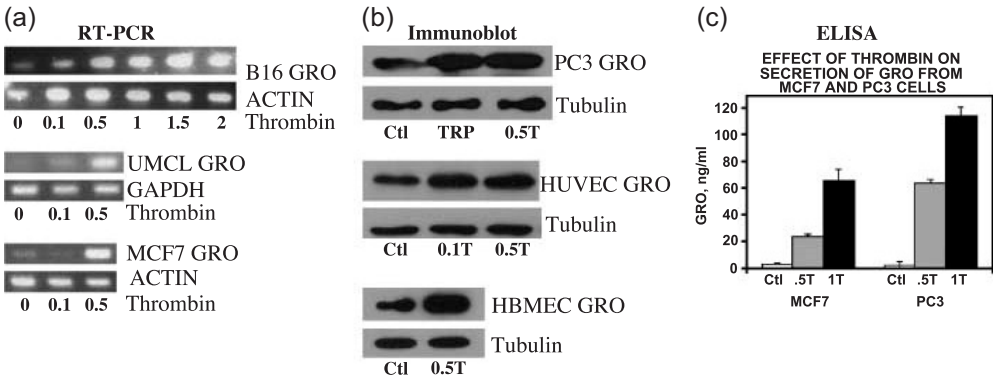

Fig. 1. Upregulation of GRO-α by thrombin. (a) Semi-quantitative RT-PCR for GRO-α in murine B16F10 melanoma, UMCL and human MCF7 breast cells. B16F10 cells were incubated with buffer 0.1, 0.5, 1, 1.5 and 2 u/ml thrombin for 24 hrs. UMCL and MCF7 cells were incubated with buffer, 0.1 and 0.5 u/ml thrombin. Actin or GAPDH was used as loading control. Representative of 2–3 experiments. (b) Immunoblot of GRO-α in PC3 cells incubated with buffer, 100 uM TRAP (TRP) or 0.5 u/ml thrombin (0.5T) for 24 hrs using tubulin as loading control. Immunoblot of HUVEC incubated with 0.1 and 0.5 u/ml thrombin. Representative of 2 experiments. Immunoblot of HBMEC incubated with 0.5 u/ml thrombin. (c) ELISA assay for GRO-α secretion in 1×10^6 MCF7 or PC3 cells at 24 hrs employing 0.5 (0.5T) and 1 u/ml (1T) thrombin. n = 3. From Caunt et al. 2006 [2].

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GRO-α is a CXC chemokine with tumor-associated angiogenic as well as oncogenic activation following ligation of its CXCR2 receptor. GRO-α enhanced angiogenesis in the chick chorioallantoic membrane (CAM) assay 2.2 fold providing direct evidence for GRO-α as an angiogenic growth factor. Anti-GRO-α Ab completely inhibited the 2.7 fold thrombin-induced upregulation of angiogenesis (Figure 2), as well as the 1.5 fold thrombin-induced upregulation of both endothelial cell cord formation in matrigel, and growth *in vitro* (Figure 3). Thrombin as well as its PAR-1 receptor activation peptide (TRAP) as well as GRO-α all markedly increased vascular regulatory proteins and growth factors: MMP-1, MMP-2, VEGF, Ang-2, CD31 and receptors KDR and CXCR2 in HUVEC. All of the thrombin/TRAP gene upregulations were completely inhibited by anti-GRO-α Ab and unaffected by irrelevant Ab (Figure 4). In vivo tumor growth studies in wild-type mice with siRNA GRO-α KD cells revealed 2-4 fold impaired tumor growth, metastasis and angiogenesis which was not affected by endogenous thrombin (Figure 5). Thus thrombin-induced angiogenesis requires the upregulation of GRO-α. Thrombin upregulation of GRO-α in tumor cells as well as endothelial cells contribute to tumor angiogenesis.

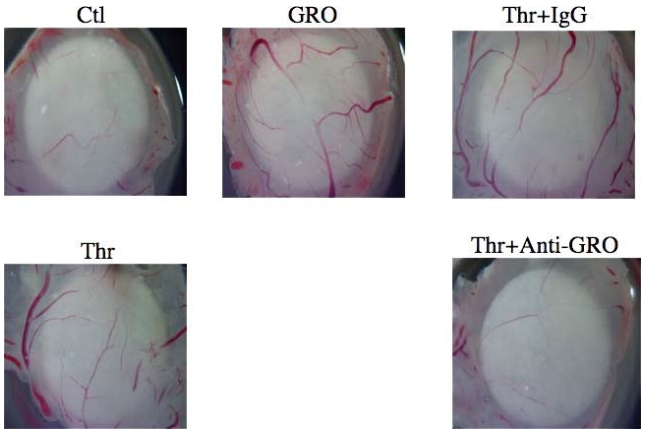

Fig. 2. Effect of GRO on angiogenesis. Display of angiogenesis on the 10 day old chick chorioallantoic membrane 72 hrs after application of PBS (Control), GRO-α, Thrombin (Thr) or irrelevant IgG or Anti-GRO-α Ab.

3. Discussion

These data clearly demonstrate a pivotal role for the chemokine GRO-α and its endothelial cell CXCR2 receptor in thrombin-induced neoangiogenesis. This was documented by analyzing the effect of both thrombin and GRO-α on the upregulation of vascular regulatory genes as well as angiogenesis in the CAM, endothelial cell cord forming and endothelial growth assays. Anti-GRO-α Ab inhibited both GRO-α as well as thrombin induced angiogenesis in the CAM, endothelial cell cord formation and endothelial cell growth assays.

Both thrombin and GRO-α upregulate: MMP-1, MMP-2, VEGF, KDR, Ang-2 and CD31 and thrombin upregulates the GRO-α receptor CXCR2. Our data indicate that this is likely to be a general response

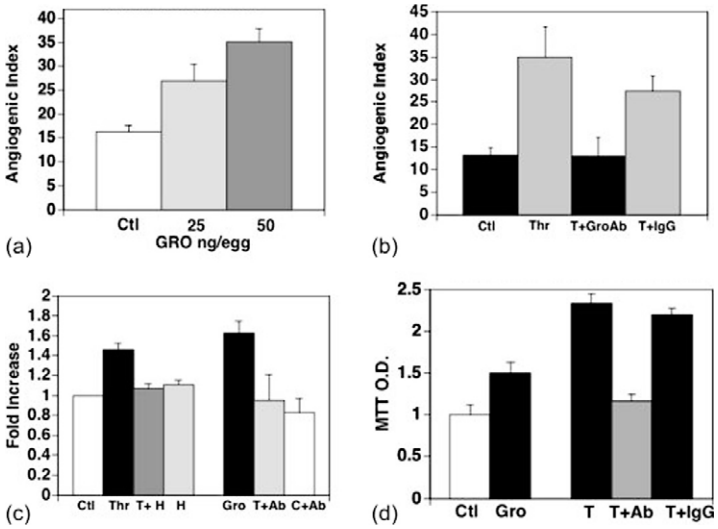

Fig. 3. Effect of GRO-α, thrombin and anti-GRO-α Ab on angiogenesis in the CAM, endothelial cord formation and endothelial cell growth assays. (a) GRO-α activation of angiogenesis at 72 hrs and 37°C in the CAM assay (25 and 50 ng/egg), n = 19. (b) Inhibition of thrombin-induced angiogenesis (Thr) by anti-GRO-α Ab (T+GROAb, 5 ug/egg) or irrelevant IgG (T+IgG), n = 9. (c) Effect of thrombin (Thr) (0.25 u/ml), thrombin plus 1 u/ml hirudin (T+H) or hirudin (H) on thrombin-induced cord formation of endothelial cells, n = 20. Effect of GRO-α (2 ug/ml), thrombin plus anti-GRO-α Ab (T+Ab, 5 ug/ml) or control plus anti-GRO-α Ab (C+Ab, 5 ug/ml) on thrombin-induced cord formation of endothelial cells in matrigel at 24 hrs and 37°C, n = 7. (d) Effect of GRO-α (0.025–0.5 ug/ml) (GRO) or thrombin (T, 0.5 u/ml) on HUVEC growth *in vitro* at 48 hrs and 37°C. Inhibition of thrombin-induced HUVEC growth in the presence of 5 ug/ml anti-GRO-α Ab (T+Ab). Absence of inhibition in the presence of irrelevant IgG (T+IgG), n = 4. From Caunt et al. 2006 [2].