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Progranulin does not inhibit TNF and Lymphotoxin α signalling through TNF receptor 1

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Running title: Progranulin does not inhibit TNFR1 signalling.

Abstract

Progranulin (Proepithelin, granulin precursor) has been recently suggested to exhibit anti-inflammatory properties by directly binding to TNF receptors and thereby inhibiting TNF signalling by Tang *et al*. This finding was challenged by Chen *et al*. and no interaction between progranulin and TNF receptor 1 or 2 was observed. We tested the ability of recombinant progranulin from different commercial sources to inhibit TNF or Lymphotoxin α induced signalling through TNFR1. We observed that progranulin does not affect signalling and cell death induction downstream of TNF or Lymphotoxin α. Our results suggest that the anti-inflammatory role of progranulin is not mediated through direct inhibition of TNF receptor 1.

Key words: Cell death, MAPK, NFκB, Progranulin, TNF receptor 1, Inflammation.

Introduction

Progranulin is a growth factor initially isolated from supernatants of the teratoma PC cell line.[1, 2] It is secreted as a full length protein and undergoes proteolysis to release small granulins. Progranulin is implicated in different physiological and pathological processes including wound repair, host defence, inflammation, tumorigenesis and neurodegeneration.[3, 4] Several studies have shown that progranulin has anti-inflammatory effects[5-7] and, *Grn*$^{-/-}$ mice display increased neuroinflammation upon injury in the CNS[8] and increased susceptibility to arthritis[9], dermatitis[10] and atherosclerosis[11] due to enhanced inflammatory responses. Tang *et al*. recently reported that recombinant progranulin or Atsttrin (a synthetic protein composed of three granulin fragments) rescued the inflammatory phenotype in a collagen induced arthritis model[9] and reduced the severity of dermatitis in an oxazolone-induced dermatitis model.[10] These observations were attributed to progranulin or Atsttrin directly binding to TNF receptors and blocking TNF signalling, which would be consistent with the known role of TNF as a master inflammatory cytokine.[12] The ability of progranulin to act as a TNF receptor antagonist was however recently challenged by Chen *et al*.,[13] who showed that progranulin does not bind to TNF receptors and therefore does not directly affect TNF signalling.

To provide some perspective to this controversy we would like to report our own results where, stimulated by the Tang *et al.* publication, we sought to test the hypothesis that progranulin (PGRN) could inhibit TNF/TNFR1 signalling differently to Lymphotoxin α (LTα)/TNFR1 signalling.

Results

Neither TNF nor LTα induced activation of NF-κB and MAP kinases is inhibited by progranulin

To test our hypothesis we examined the ability of progranulin (PGRN) to inhibit activation of NF-κB and MAP kinases by either TNF or LTα, two ligands that bind and signal via TNFR1.[14] To test the reported functionality of PGRN, we treated wild-type bone marrow derived macrophages (BMDMs) for up to 2 hours with TNF or LTα in the presence or absence of recombinant human PGRN (Figure 1a). Analysis of well described signalling outcomes upon TNF or LTα stimulation including ERK, JNK and p38 phosphorylation and IκBα degradation did not reveal any inhibition by PGRN. Because this result did not agree with the result described by Tang *et al*. we tested human and mouse PGRN from a different commercial source (Adipogen), which was reported to be a reliable source for functional PGRN.[15] However, regardless of the source or the species of PGRN, we did not observe any inhibition of TNF-induced ERK phosphorylation or IκBα degradation (Figure 1b). In contrast, a TNFR1 blocking antibody was very efficient at blocking these pathways showing that this assay accurately reported on TNFR1-dependent signalling pathways.

While these initial results excluded a role of PGRN on transient TNF signalling, it was possible that PGRN affected TNFR1 signalling only over a longer time period. To test this hypothesis, we examined the long-term effects of all three PGRNs on the human leukemic cell line U937, which was stably transfected with a NF-κB-GFP reporter. Consistent with our previous results we did not observe any reduction in NF-κB activation after 24 hours TNF or LTα stimulation by either of the three tested PGRNs (Figure 1c&d).

Progranulin does not inhibit TNF or LTα dependent apoptosis and necroptosis

Loss of cellular signalling components such as IAPs converts the pro-survival signal of TNFR1 to a cell death-inducing stimulus.[16] To test whether progranulin could interfere with TNF or LTα induced apoptosis, we pre-treated U937 cells with three different sources of PGRN at various PGRN to TNF ratios before stimulating them

with either TNF or LTα in combination with smac mimetic (SM) for 48 hours (Figure 1e). We did not observe any difference in cell death in the presence or absence of PGRN regardless of the TNF to PGRN ratio (data not shown).

Upon caspase inhibition, engagement of TNFR1 can result in alternative form of cell death, termed programmed necrosis (necroptosis).[17] As an additional, independent read-out of TNF signalling in another cell type we therefore examined the effect of PGRN on apoptosis and necroptosis induced by TNF or LTα in mouse dermal fibroblasts (MDFs, Figure 1f). In agreement with our previous results, apoptosis, where caspases are active and necroptosis, where caspases were inhibited by pan caspase inhibitor (QVD), induced by TNF or LTα and smac mimetic were unaffected by PGRN treatment.

Discussion

PGRN can function as an anti-inflammatory molecule, however the mechanism for the anti-inflammatory action of PGRN is unknown. For this reason the report by Tang *et al.* demonstrating that PGRN directly inhibited TNF binding to, and inflammatory signalling from, TNFR1 and TNFR2 provoked intense interest. To extend the findings of Tang *et al*. we sought to determine whether PGRN could inhibit TNF/TNFR1 signalling differentially to LTα/TNFR1 signalling. However, in contrast to the results published by Tang *et al*. we were unable to observe any effect on either TNF or LTα induced signalling in various cell types in either acute or chronic signalling scenarios with three types of progranulin from two commercial sources. In our studies we used different cell types from both mouse and human, to exclude cell type or species-specific effects. In contrast to the results from Tang *et al.* we were unable to observe any inhibition of TNFR1 induced signalling: the timing and degree of IκBα degradation and phosphorylation of ERK, JNK and p38 were all indistinguishable regardless of whether cells were pre treated with PGRN or not. While in the same experiments anti-TNFR1 was able to completely block these signalling events. Furthermore, TNF induced apoptosis or necroptosis were not inhibited by any of the progranulins tested. These results are consistent with those published by Chen *et al.* who also tested a number of different PGRNs in a range of TNF dependent assays but failed to detect any inhibition of TNF signalling.[13]

Our assays were performed under the assumption that the identity of our commercially sourced recombinant progranulin is correct, an issue that was discussed by *Tang et al*.[15] We controlled this assumption using both Western blot and mass spectrometry and we confirmed that the recombinant progranulins contained a single species that ran at the correct size that was detected with an anti-progranulin antibody and that contained tryptic progranulin peptides (data not shown). However this analysis cannot determine whether the recombinant progranulin is correctly folded. However, Atsttrin, a recombinant molecule synthesised from 3 non-contiguous granulin domains, was reported to have the same biological effects as PGRN by Tang *et al*. This indicates that higher order structure of PGRN is not critical for its purported activity.

It was possible that high endogenous levels of PGRN in the cell culture supernatants could interfere with the action of recombinant PGRN. However we did not detect any progranulin in cell lysates or supernatants of MDF and U937 cells (data not shown). BMDMs secreted low levels of PGRN in the supernatant, however as the signalling kinetics were identical in all cell types tested, and levels of endogenous PGRN significantly below the levels added ectopically it is unlikely that endogenous PGRN saturates TNF Receptors preventing recombinant progranulin from inhibiting further.

Our results support those described by Chen *et al.* and contradict those of Tang *et al*. Outside of the explanations described above we cannot explain why we were unable to repeat the observations of Tang *et al*. Regardless of the explanation, our results suggest that PGRN inhibition of TNF signalling is unlikely to be easy to observe with commonly available reagents and suggest that caution should be used before trying to develop reagents based on PGRN for clinical and preclinical studies.

Methods

Cell culture and reagents

All cells were maintained in DMEM + 10% FCS. MDFs and BMDMs were generated using a previously described protocol.[14] Stable NF-κB-GFP reporter U937 cells were generated using the NF-κB lentiviral reporter vector pTRH1 mCMV NF-κB dscGFP from System Biosciences. All tissue-harvesting procedures were performed according to the guidelines of the Animal Ethics Committee of the Walter and Eliza Hall Institute of Medical Research (WEHI).

Ligands and compounds

Fc-TNF and Fc-LTα were produced and purified as described and used at a concentration of 10 ng/ml (for BMDMs, U932) or 100 ng/ml (for MDFs).[18] The smac mimetic, Compound A, has been previously described[16], was synthesised by TetraLogic Pharmaceuticals and used at a concentration of 500 nM. Pan caspase inhibitor, QVD (10 μM; MP Biomedicals) was added 1 hour prior to TNF or LTα and smac mimetic treatment. Recombinant progranulins were purchased from R&D Systems (human; 2420-PG-050) and Adipogen (human; AG-40A-0188, mouse; AG-40A-0080) and used at 250 ng/ml. Monoclonal TNFR1 and 2 antagonistic antibodies were purchased from R&D systems (MAB430, MAB4262) and used at a concentration of 2 μg/ml.

Death assay and flow cytometry

Cells cultured in 12 or 24 well tissue culture plates were harvested 24 or 48 hours after stimulation with TNF or LTα/smac-mimetic/Q-VD-Oph and cell death was measured by Propidium Iodide (PI) staining and flow cytometry on a FacsCalibur (BD Biosciences). Data processing was performed using Weasel software (WEHI).

Western blotting and antibodies

Cell lysates were prepared using DISC lysis buffer as described before[14] , loaded on NuPAGE Bis-Tris gels (Life Technologies) and transferred on to Immobilon-P PVDF (Millipore) or Hybond-C Extra Nitrocellulose membranes (GE Healthcare).

Membranes were blocked and antibodies diluted in 5% skim milk powder or 5% Bovine Serum Albumin (BSA) in PBS and 0.1% Tween20. Antibodies used for Western blot: phospho-ERK1/2, phospho-JNK1/2, phospho-p38, p38, ERK1/2, JNK1/2, IκBα (Cell Signaling), β-actin (Sigma Aldrich). Signals were detected by chemiluminescence (Millipore) after incubation with secondary antibodies conjugated to HRP.

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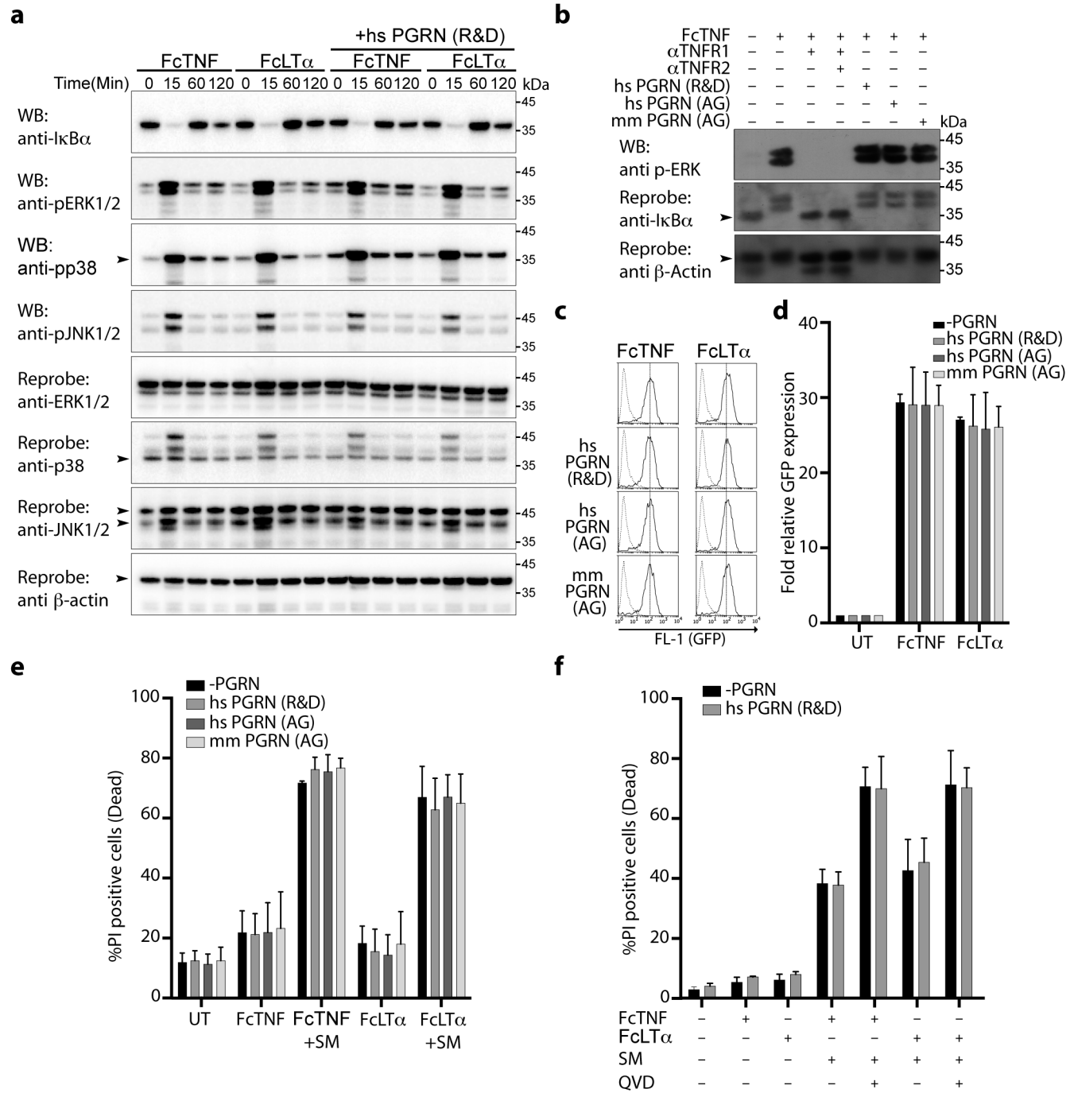
Figure legends

Figure 1. Progranulin does not inhibit TNF receptor 1 signalling. (a) BMDMs were pre-treated with human PGRN (R&D Systems) for 30 minutes and stimulated as indicated with TNF or LTα. Cell lysates were analysed by Western Blot for degradation of IκBα and phosphorylation of ERK, JNK and p38. (b) BMDMs were pre-treated with human or mouse PGRN (R&D Systems, Adipogen) or anti TNFR1 and TNFR2 as indicated. Cells were then stimulated with TNF for 15 minutes and cell lysates were analysed by Western Blot for degradation of IκBα and phosphorylation of ERK. (c,d) U937 cells stably transfected with a NFκB-GFP reporter were pre-treated with indicated PGRNs and stimulated with TNF or LTα. 24 hours after stimulation, expression of GFP was assessed by flow cytometry. (e) U937 Cells were pre-treated with PGRNs followed by addition of TNF or LT with or without smac mimetic compound (SM) for 48 hours. (f) MDFs were pre-treated with human PGRN (R&D) and then stimulated with TNF or LTα with or without smac mimetic (SM) for 24 hours. Cell death was assessed by PI staining and flow cytometry. Error bars=SD; n=3.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WB: anti-pJNK1/2
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Reprobe: anti-JNK1/2
Reprobe: anti β-actin
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WB: anti p-ERK
Reprobe: anti-IκBα
Reprobe: anti β-Actin
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