Selective internalisation of a novel recombinant human granzyme B by membrane Hsp70 positive tumour cells and its cytotoxic consequences: A novel therapeutic approach for cancer?

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Immune effector cells such as natural killer (NK) cells and cytotoxic T cell subsets can induce the apoptosis of target cells via the delivery of serine proteases. Perforin released from cytotoxic granules facilitate the uptake of cytotoxic mediators, although the precise mechanisms remain unclear. Tumour cells have been shown to selectively express a membrane form of the 70 kDa heat shock (stress) protein Hsp70 which acts as a recognition structure for activated natural killer (NK) cells [1-3]. Furthermore, it has been shown that the membrane-expressed form Hsp70 exhibits a high turn-over rate on the cell surface [4] and can also facilitate the selective uptake of granzyme B into membrane Hsp70 positive tumor cells *in vitro* to result in the induction of perforin-independent apoptosis [5].

As part of a continuing programme which is aimed at further investigating the potential of granzyme B as a therapeutic which can target membrane Hsp70 positive tumours using *in vivo* human xenograft and murine syngeneic tumor mouse models, we have developed a human expression system for the production of high yields of enzymatic and biologically active human granzyme B [6]. For this, human embryonal kidney cells (HEK293) were stably transfected with human granzyme B. The HEK293 host cells were protected from apoptotic cell death by fusing an inactivation site coupled to a (His)6 tag to the gene sequence of granzyme B. Inactive granzyme B which was actively released from HEK293 cells by insertion of a Igκ leader sequence was purified on a nickel column utilising the (His)6 tag. After enterokinase digestion and heparin affinity chromatography, high yields of enzymatic and biologically active human granzyme B were obtained [6]. The current study used confocal microscopy to investigate the internalisation of granzyme B into membrane Hsp70 positive tumour cells (the human breast carcinoma derived MCF7, the murine colon carcinoma line CT26 and the murine breast carcinoma line 4T1) using granzyme B that was conjugated to Atto488 using Lightning-Link® labelling kits (Innova Biosciences Ltd., Cambridge, UK) and its cytotoxic consequences. Atto488-conjugated bovine serum albumin was used as a control.

The incubation of membrane Hsp70 positive tumour cell lines (MCF7, CT26 and 4T1) with granzyme B induced apoptosis, as was illustrated by morphological changes and the activation of caspase-3, which was first apparent after 4 hours and progressed to up to 100% cell death after 24 hours. The interaction of granzyme B with membrane Hsp70 required glycosylation, as binding and uptake of deglycosylated granzyme B was much reduced, as assessed by flow cytometry and confocal microscopy. Binding and uptake were also inhibited by incubation with neuraminic acid, thereby suggesting that perforin-independent interaction of granzyme B with membrane Hsp70 positive tumor cells is associated with mammalian glycosylation and is mediated by the oligosaccharide moiety of neuraminic acid (NANA). Current work is focussing on better understanding the intracellular movement of granzyme B through endosomes and lysosomes by co-staining cells with antibodies specific for endosomal (early with Rab-4 and -5, late with Rab-7 and -9, recycling with Rab-11, -15, -17 and -25) and lysosomal compartments (LAMP1 and LAMP2) compartments, and MitoTracker Green dye to identify the mitochondria.

In conclusion, these findings indicate that granzyme B could offer a new therapeutic option for the targeting cancer in the large proportion of patients that bear membrane Hsp70 positive tumours.

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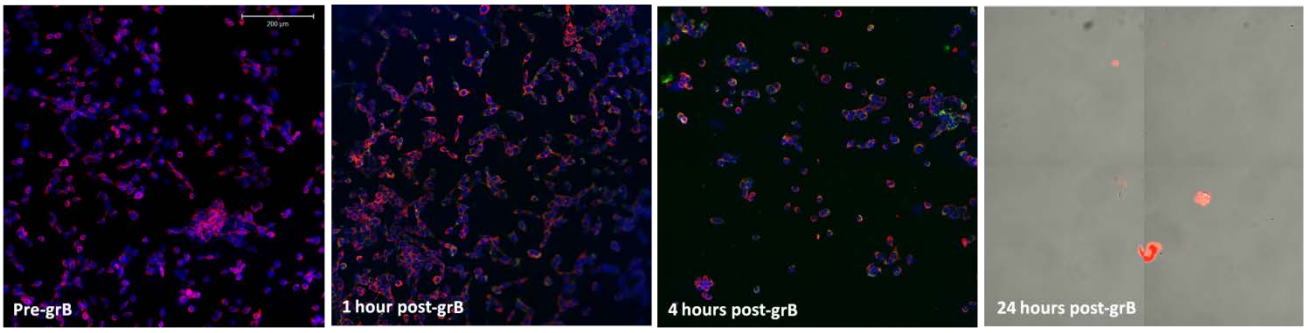

Figure 1. *CT26 cells grown in MatTek glass bottom dishes for 48 hours, then received growth media + 4ug/ml grB in place of the current growth media. Images were taken using a Zeiss 510 Inverted Microscope immediately before adding granzyme B ("Pre-grB") and 1,4 and 24 hours subsequently.Cells can be seen to start rounding up by 4 hours, whilst after 24 hours all cells have detached from the plate.*

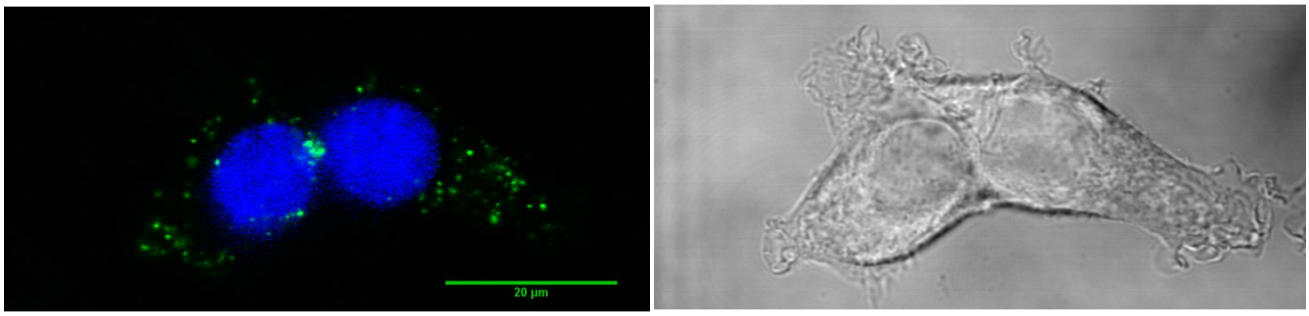

Figure 2. *Confocal image of a 700nm slice in the mid-plane through CT26 cells, showing internalisation of granzyme B (green) after 2 hours. At this time granzyme B looks to be localised in vesicles and there does not appear to have been any trafficking of granzyme B as far as the nucleus (shown in blue by DAPI staining).*