Bioactivity of a multivalent cell membrane binder in 3D spheroid culture: effects of RGD-dendrimer conjugate on cell proliferation, expression and aggregation

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Statement of Purpose: By presenting biomolecular ligands on the surface in high density, ligand-decorated dendrimers are capable of binding to membrane receptors and cells with specificity and avidity. However, the current investigations on ligand-dendrimer conjugates have mainly focused on their binding behavior with cells, whereas their potential bioactivity in multicellular systems, especially in three-dimensional (3D) culture systems, remains untapped. In this study, a widely used adhesive tripeptide ligand—RGD—was modified to polyamidoamine (PAMAM), and the bioactivity of suspended RGD-PAMAM conjugates was investigated on cells cultured as multicellular spheroids *in vitro*. Our results demonstrate that the RGD-PAMAM conjugate was able to promote cellular proliferation and aggregation and affect the mRNA expression of many extracellular factors. These bioactive functions of RGD-PAMAM were multivalency-dependent, as none of similar effects was observed for the monovalent RGD ligand. Our study suggests that multivalent ligand-dendrimer conjugates may act as unique artificial factors to affect the cellular microenvironment in 3D culture and would find various applications in 3D cell culture and tissue engineering.

Methods: To synthesize RGD-PAMAM, G4.0 PAMAM was activated with a heterobifunctional crosslinker, N-hydroxysuccinimide-PEG2-maleimide, $SM(PEG)_2$ and a cysteine terminated sequence CGRGDS was covalently conjugated to the PAMAM dendrimer. Spheroids of NIH 3T3 fibroblasts were induced and cultured using 24-well ultra-low attachment plates (ULAPs) in the presence of RGD-PAMAM of varied concentrations (10-100 μM). Fluorescein-labeled RGD-PAMAM (f-RGD-PAMAM) was used to visualize the conjugate material in culture. The cellular viability was studied by MTS assay and the DNA contents were quantitatively determined by PicoGreen measurement. The mRNA expression levels of a series of extracellular factors were quantified using the SYBR Green real-time PCR. Cell aggregation was studied by the morphology of spheroids and the quantitative analysis of spheroid size and number in 3D culture through optical and confocal fluorescence microscopy.

Results: A RGD-PAMAM dendrimeric conjugate with a peptide conjugation level (52%) versus the peripheral was used in this study. RGD-PAMAM was able to bind with cells in 2D monolayer and 3D spheroid culture (Fig A). The metabolic activity level and DNA quantitation at 24 h both indicate a significant increase of cell proliferation upon incorporation of RGD-PAMAM into the spheroid culture. The cellular response seemed to be concentration-dependent, with higher conjugate concentration inducing higher level of proliferation. RGD-PAMAM was also found to significantly upregulate the mRNA levels of growth factors including bFGF, HGF and TGF-β while decrease the expression of adhesive proteins ─collagen and fibronectin. (Fig B) In contrast, monovalent ligands or $SM(PEG)_2$-PAMAM conjugates themselves did not show any effects on the spheroid culture. In aggregation studies, RGD-PAMAM was found to promote the size of multicellular spheroids in a concentration dependent manner. When the distribution of spheroid diameters was measured and compared, the diameter of spheroids of highest population grew significantly from 50-60 μm in non-treated samples to 130-140 μm in samples treated with 100 μM RGD-PAMAM at 48h. (Fig C) It is speculated that within the constructs of multicellular spheroids, the dendrimeric conjugate molecules with nanospherical shapes would get exposed to multiple cells and function as a "gluing" material. A model was proposed to illustrate the cell aggregation was promoted through the interaction of RGD-PAMAM with membrane integrin receptors of different cells. (Fig D)

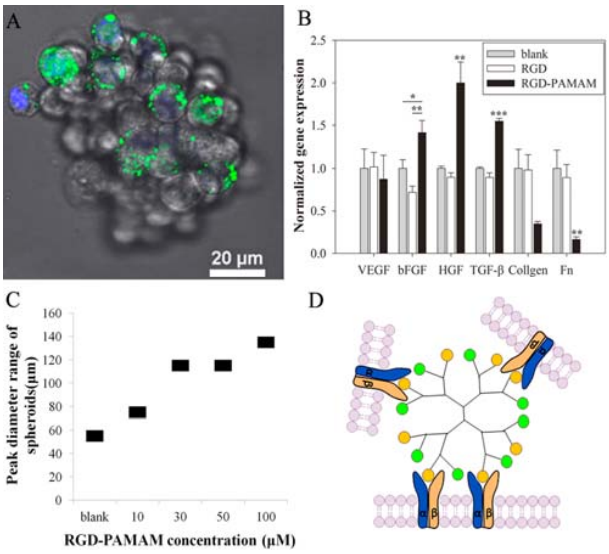

Fig.1. RGD-PAMAM binds to cell membranes in cellular spheroids (A), qPCR of mRNA expression (B), comparison of diameter range of spheroids of highest population at 48h (C) and a scenario model of RGD-PAMAM interacting with multiple cells through integrin receptors (D).(n= 3; *, **, and ***, p values of <0.05, 0.01, and 0.001, respectively)

Conclusions: Multivalent adhesive conjugates were incorporated into the multicellular aggregates in a dosage controllable manner. Under 3D culture conditions, the RGD-PAMAM conjugates were capable of promoting multicellular aggregation and enhancing the cellular activity and function *in vitro*. Ligand modified dendrimers therefore may have potential for use as soluble functional nanomaterials to provide new methodologies for 3D cell culture and tissue engineering.

References: Zhao D. Biomaterials. 2008;29:3693-702; Fasting C. Angew Chem Int Ed Engl. 2012;51:10472-98.