Cell Survival Responses Regulated through Controlled Nanoscale Presentation of EGFR and Integrin Ligands

Eileen T. Dimalanta[1], Maria L. Ufret[1], Llewellyn B. Richardson[1], Nicholas A. Marcantonio[1], Benjamin D. Cosgrove[1], Ikuo Taniguchi[1,2], William Kuhlman[2], Anne M. Mayes[2], Linda G. Griffith[1].

[1]Division of Biological Engineering, [2]Department of Materials Science and Engineering
Massachusetts Institute of Technology.

Statement of Purpose: The epidermal growth factor receptor (EGFR) has an array of physiological ligands, many of which are bound to or embedded in extracellular matrix. Matrix-bound ligands can induce differential cell responses in the physiological setting, as seen in the recent report of the protective effects of amphiregulin on liver cell survival under liver injury *in vivo*[1]. Delivering EGFR ligands in a manner that restricts signaling to the cell surface may thus be a useful approach for certain tissue engineering applications, but little is known about the signaling mechanisms and downstream cellular responses.

To present EGF as a ligand that elicits surface-restricted EGFR signaling from the culture substrate, we have used a comb copolymer system that comprises a hydrophobic backbone with 30-100 PEO side chains spaced < 2 nm apart. These polymers form thin transparent films and present a PEO brush interface when cast on glass substrates. The PEO chain ends can readily be activated to react with cysteines or primary amines. If a small fraction of activated comb is added to an excess of inert comb, and the resulting film reacted with the ligand of interest, the substrate presents islands of highly clustered ligands against an inert background. This presentation mode is essential to retaining the activity of EGF, as dimerization of the EGFR is required for signaling, and the polymer presents the ligand in a spatial arrangement conducive to EGFR homodimerization.

Further, we have developed a means to present the tethered EGF ligand against a well-defined adhesion background by mixing comb polymers modified to react with cysteines with polymers modified to react with amines, allowing us to co-tether an adhesion ligand via a terminal cysteine and EGF via the terminal amine. As we are particularly interested in adhesion mediated by the a5b1 integrin, we used a new synthetic adhesion ligand with high affinity for a5b1 to assess the effects of tethered EGF molecules on EGFR phosphorylation and downstream signaling in hepatocytes against a defined adhesion background. Further, we found that tethered EGF elicited differential survival effects in cells challenged with an apoptotic stimulus.

Methods: The comb copolymer (PMMA-PEO copolymer) was synthesized as previously described[2] The copolymer was activated with N-(p-maleimidophenyl) isocyanate (PMPI) or 4-nitrophenyl chloroformate (NPC), which reacts with the hydroxyl end of the PEO side chains, and allows for tethering of a ligand via sulfhydryl group (cysteine) or primary amine, respectively. Bifunctional surfaces (targeting both a5b1 integrin and EGFR) were made by covalently linking PHSRN-RGD (a synthetic peptide targeting a5b1) and EGF to comb copolymer coated glass cover slips. Primary rat hepatocytes were isolated utilizing a collagenase perfusion method from 150-230 gram male Fischer rats[3]. EGFR phosphorylation and downstream signaling was studied using fluorescence microscopy and Western blot analysis. The effect of tethered EGF on cellular response to death stimuli was studied using flow cytometry.

Results / Discussion: The novel adhesion ligand, PHSRN-RGD, was developed to specifically target the a5b1 integrin, which adheres poorly to RGD. This peptide greatly enhanced adhesion of cells expressing the a5b1 integrin. A bifunctional surface with PHSRN-RGD and EGF results in an adhesive surface that stimulates the EGF receptor on primary rat hepatocytes. Tethered EGF activated the receptor, as detected by phosphorylation at multiple sites (tyrosines 845, 1068, and 1173). Cell signaling via the receptor activated both ERK and AKT, molecules involved in pro-survival pathways. In addition, fluorescence micrographs show that signaling of the EGF receptor on tethered EGF substrates was restricted to the cell surface, inhibiting the internalization and downregulation of the receptor.

Rat hepatocytes were incubated with adenovirus serotype 5 carrying the gene for enhanced green fluorescent protein (EGFP), which induces significant apoptosis at MOI ~10. Cells cultured on surfaces with tethered EGF had a lower degree of apoptosis compared to cells cultured with soluble EGF in the growth media.

Conclusions: The bifunctional surface developed in this lab targets both integrins and the EGF receptor, creating a well defined environment for cells by spatially controlling the presentation of ligands on a nanometer length scale. PHSRN-RGD provides a robust adhesion ligand for cells expressing a5b1, resulting in surfaces amenable for studying the effects of co-tethered growth factors in signaling pathways. Tethered EGF was shown to signal via the EGF receptor, activating downstream molecules involved in pro-survival pathways. Moreover, the receptor bound to tethered EGF was inhibited from internalizing. It is possible that this inhibition causes prolonged signaling of the receptor, resulting in decreased levels of apoptosis of hepatocytes infected with the adenovirus. The interaction between growth factor receptors, integrins, and their ligands is a complicated one due to the multitude of ligands, receptors, and growth factors present in tissue. Our ability to control the spatial presentation of ligands and growth factors provides a practical platform for parsing cell signaling pathways. Such a platform could prove invaluable for uncovering aspects of signal transduction important to the design of *in vivo* therapies and to manipulate cell responses for directed tissue regeneration.

References:

[1] Berasain c. J Biol. Chem. 2005 (280:19012-19020).
[2] Irvine DJ. Biomacromolecules 2001 (2:84-95).
[3] Powers MJ Biotechnol. Bioeng. 1996 (50: 392-403).