Published in final edited form as:

J Biomed Mater Res A. 2009 November ; 91(2): . doi:10.1002/jbm.a.32214.

“Methods to promote Notch signaling at the biomaterial interface and evaluation in a rafted organ culture model”

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Abstract

The Notch signaling pathway is a promising target for controlling cell fate choices at the biomaterial-tissue interface. Building on our previous work in developing Notch-signaling biomaterials, we evaluated various immobilization schemes for Notch ligands and their effect on human foreskin keratinocytes. A peptide sequence derived from the Jagged-1 DSL-region and immobilized to poly (2-hydroxyethyl methacrylate) (polyHEMA) showed no bioactivity in relation to the Notch-CSL pathway. The full-length Jagged-1 protein immobilized directly to the polyHEMA surface showed activity in signaling the Notch-CSL pathway. However, an indirect affinity immobilization approach yielded a stronger signal. Human keratinocytes plated on bound Jagged-1 showed upregulated involucrin, keratin 10, and loricrin protein expression, with this expression being cell density-dependent. Utilizing a human foreskin rafted organ culture model as a bridge between *in vitro* and *in vivo* studies, Jagged-1-modified or control polyHEMA rods were implanted in human foreskin and cultured at the air-medium interface. Keratinocyte proliferation was suppressed and intermediate-stage differentiation promoted in Jagged-1-modified rods compared to control rods. Thus, Notch-signaling biomaterials provide a robust approach to control keratinocyte differentiation and may find application to other progenitor and stem cells.

Introduction

Cells tend to interact with biomaterials in a non-specific manner and the endpoint for all biomaterials is essentially equal: contraction in a collagenous scar.¹ However, by employing bioactive surface modifications, a specific rather than non-specific cellular response may be elicited. Bioactive scaffolds have been employed in the tissue engineering, regenerative medicine, and medical device fields to improve host response and provide an additional therapeutic benefit to the biomaterial.² Another important goal is to control cell fate at the device interface. This is particularly important as we begin to identify specific signaling pathways that control stem and other progenitor cell differentiation programs.

We have previously described a system to control epithelial differentiation at the biomaterial interface using immobilized Jagged-1, a Notch ligand.³ Notch is normally a cell-to-cell signaling receptor that is active in numerous cell fate decisions.⁴ Its role in cell fate decisions makes Notch a particularly promising target for guiding stem cell and progenitor

cell differentiation in tissue engineering applications. Numerous studies have provided initial promise for harnessing Notch to control regeneration and stem cell fate.⁵⁻⁷ The Notch receptor and its ligands (Delta-like 1, 3, 4 and Jagged-1,2) are both type I transmembrane proteins.^{8,9} Upon binding of the Notch receptor by its ligand, a cascade of proteolytic events is initiated, eventually leading to release of the Notch cytoplasmic domain (Notch^{IC}). In the canonical pathway, this cytoplasmic domain translocates to the nucleus and converts Centromere binding protein 1 (CBF-1) from a transcriptional inhibitor to activator.⁸ As with all signaling pathways, Notch signaling is regulated and influenced by a myriad of conditions. For example, a soluble ligand can either serve as a Notch agonist or antagonist depending on the context.^{4,10-12} In addition, there has been some indication that a peptide sequence derived from the Delta/Serrate/Lag2 (DSL)-region of the ligand can influence Notch signaling.¹³⁻¹⁵

As previously published by our group, rat esophageal epithelial cells (REEC) plated on biomaterial-immobilized Jagged-1 initiated the canonical Notch/CSL signaling pathway.³ Signaling resulted in cells rapidly (<72hrs) upregulating both intermediate- (involucrin and keratin 10) and late-stage (filaggrin) differentiation markers, in addition to tight clustering and stratification of the cells.³ While these studies implicated Notch in the control of epithelial stem cell differentiation, the effect of biomaterial-based Notch signaling in human epithelial cells *in vitro* or *in vivo* was not determined.

Recently, Knowles et al. described an *in vitro* rafted organ culture (ROC) model that mimics the *in vivo* human keratinocyte response to a biomaterial in an *in vitro* setting.^{16,17} In this model, a biomaterial rod is inserted through a 4mm punch biopsy of human neonatal foreskin tissue and cultured at the air-medium interface. For our purposes, the ROC model allows us to approximate the epithelial response to a Jagged-1-modified surface. The system is advantageous in that the organ culture provides a full range of cell types, allows a variety of biomaterials to be employed, eliminates influence of any immune response, and focuses on the epidermal/biomaterial interaction. However, it does have limitations of being an *in vitro* system with no blood supply or innervation. In addition, it provides only a partial understanding of the body's true response due to the lack of an immune response or biomaterial integration by non-epidermal cell types. Understanding these limitations, we employed the ROC model as a bridge to a full *in vivo* study, thus allowing us to elucidate the human keratinocyte response to a Notch-stimulating biomaterial.

In this paper, we have further developed the conditions required to achieve Notch signaling at the biomaterial interface. Various immobilization schemes (direct and indirect) and ligands (full-length Jagged-1 or DSL-region based peptide) were evaluated for their ability to signal the Notch/CBF-1 pathway. In addition, the effect of Jagged-1 on human neonatal foreskin keratinocyte differentiation was analyzed. Finally, the modified biomaterial was tested in the organ culture model as an initial step towards understanding the possible epithelial responses *in vivo*.

Materials and Methods

Cell culture

Neonatal human keratinocytes (HEK) were purchased from Cascade Biologics (Portland, OR) and cultured in EpiLife basal medium (0.06mM Ca⁺⁺, Cascade Biologics) and supplemented with human keratinocyte growth supplement (HKGS, Cascade Biologics). The cells were cultured according to manufacturer's instructions. REECs were isolated and cultured as previously described.^{3,18}

Jagged-derived peptide

The peptide based on the Jagged-1 DSL region (Jag_DSL) and the scrambled control (Sc_Jag) were synthesized by SynPep (Dublin, CA). The amino acid sequence for Jag_DSL was: CDDHYYGFGCNKFCRPRGGGGGGGK-COOH, derived from the previously reported sequence.^{14,19} The scrambled peptide (Sc_Jag) had the sequence RCGPDCFDNYGRYKHCFGGGGGGGK-COOH, and was based on a previously used scrambled sequence.¹⁴ Both peptides had a purity >90% and a measured mass of 2551 daltons.

PolyHEMA preparation

Poly (2-hydroxyethyl methacrylate) (polyHEMA) surfaces and scaffolds were prepared using a variety of methods.^{3,20-22} Spin cast polyHEMA surfaces were prepared by first spin coating the coverglass (10mm dia) with EMA-silane (2.5% in ethyl acetate) followed by spin casting polyHEMA (2.5% in methanol, Polysciences, Warrington, PA).^{3,21} Non-porous polyHEMA was prepared by polymerizing sheets of polyHEMA between two glass plates separated by polytetrafluoroethylene (PTFE) spacers (5mm) using formulation I as reported by Ratner and Miller.²² Porous polyHEMA was prepared using a sphere-template method developed by Marshall, et al.²⁰ Briefly, sifted and close-packed poly(methyl methacrylate) (PMMA) beads (61-75µm dia, Proflo Crystal Clear Power, Kupa Inc., Buena Park, CA) were sintered together between glass plates for 20hrs at 139°C to produce necking at 40% of the bead diameter. A solution of HEMA monomer (same formulation as above) was then infiltrated into the PMMA bead cake and polymerized in the interstices. After polymerization, the PMMA beads were solubilized with acetone, leaving behind a porous polyHEMA scaffold with a highly regular pore structure.²⁰

Protein/peptide immobilization

Immobilization of the proteins/peptides to the surface of polyHEMA utilized 1,1 carbonyldiimidazole (CDI) chemistry.^{3,21,23} The hydroxyl groups on polyHEMA were reacted with CDI (20mM in acetone, Sigma, St. Louis, MO) for 2.5hrs at 50°C to produce an active intermediate carbamate at these sites. After acetone rinses, the protein/peptide was added to the polyHEMA and allowed to react overnight at 4°C in a 0.1M sodium bicarbonate buffer (pH 9.3). The final product was the protein/peptide immobilized through their primary amines to the hydroxyl groups on polyHEMA with a carbonyl spacer introduced by the CDI.²³

Surface analysis and scanning electron microscopy

Electron Spectroscopy for Chemical Analysis (ESCA) was performed at the National ESCA and Surface Analysis Center for Biomedical Problems (NESAC/BIO, funded by the National Institute for Biomedical Imaging and Bioengineering of the National Institutes of Health, EB-002027). Data were collected using a Surface Science Instruments (SSI) S-Probe as previously described.²¹ For scanning electron microscopy (SEM) characterization, samples were lyophilized and then sputter coated for 80sec at 3Å/sec (SPI MODULE™ sputter coater, West Chester, PA) and imaged using a FEI Sirion XL-30 SEM.

CBF-1 luciferase assay

Activation of Notch signaling by the biomaterial immobilized ligands was measured using a previously described CBF-1 luciferase assay.^{3,12} In this assay, cells were transiently transfected (Fugene 6, Roche Diagnostics, Switzerland) with 900ng of a firefly luciferase plasmid containing four adjacent CBF-1 binding sequences (gift from L. Liaw, Main Medical Center Research Institute). Included in the transfection was 100ng of *Renilla* SV40 construct (Promega Corporation, Madison, WI) as a transfection efficiency control. The

following day, these cells were passaged and replated on the immobilized ligand (medium contained 1.5mM Ca^{++}). After 24hrs, the protein was collected and luciferase content analyzed using the Promega Dual-Luciferase® Reporter Assay System (Promega). Results are reported as the average firefly to *Renilla* ratio and normalized to the Fc control.

Quantification of Adsorbed Protein and Notch Activation

For quantification of direct and indirect immobilization of Jagged-1/Fc, a Micro-Bicinchoninic acid (BCA) Protein Assay was used. For direct immobilization, a 96-well plate was coated overnight with Jagged-1/Fc at varying concentrations (0, 1, 10, 30, and 300nM). These wells were rinsed well with PBS and then another 25µl of PBS was added to the wells. A Jagged-1/Fc standard curve was generated by adding varying concentrations of Jagged-1/Fc diluted in PBS (0-8µg/ml) to separate wells (25µl). For indirect immobilization, wells were incubated overnight with 10µg/mL of rabbit anti-Human Fc. These wells were then rinsed and varying concentrations of Jagged-1/Fc (0, 1, 10, 30, and 300nM) were added and incubated for 2hrs. These wells were rinsed again and another 25µl of PBS added. The standard curve was generated for anti-Fc (0-16µg/ml) and anti-Fc (4µg/ml) + Jagged-1/Fc (0-8µg/ml) diluted in PBS (final volume 25µl). This allowed for subtraction of the anti-Fc protein adsorbed to the surface and generation of a Jagged-1/Fc standard curve in the presence of anti-Fc. Both direct and indirect immobilized samples were reacted for 1hr with the MicroBCA Protein Assay Reagent (25µl, final volume 50µl) according to manufacturer's protocol and absorbance read at 562nm. Quantification was based on relating absorbance of the standard curve to the absorbance of the immobilized protein.

To measure Notch activation, a CBF-1 luciferase assay was performed as outlined above. Briefly, for direct immobilization, 48-well TCPS plates were treated overnight with varying concentrations of Jagged-1/Fc or human Fc. These wells were subsequently blocked for 2hrs with BSA (10mg/ml in PBS). For indirect immobilization, plates were treated overnight with rabbit anti-Human Fc (15µg/ml in PBS, Jackson ImmunoResearch, West Grove, PA), blocked 2hrs at room temperature with bovine serum albumin (BSA, 10mg/ml in PBS, Fraction V, Sigma), and finally treated with either human Fc (Jackson) or Jagged-1/Fc (R&D Systems, Minneapolis, MN) at 30nM diluted in PBS with 1mg/ml BSA for 2hrs at room temperature. Plates were rinsed with PBS between each step for both indirect and direct immobilizations. CBF-1 luciferase assays were conducted using HEK as outlined above.

Differentiation studies

To analyze the effect of bound Jagged-1 on HEK differentiation, 12-well tissue culture polystyrene (TCPS) plates were treated overnight with rabbit anti-Human Fc (15µg/ml in PBS, Jackson ImmunoResearch, West Grove, PA), blocked 2hrs at room temperature with bovine serum albumin (BSA, 10mg/ml in PBS, Fraction V, Sigma), and finally treated with either human Fc (Jackson) or Jagged-1/Fc (R&D Systems, Minneapolis, MN) at 30nM diluted in PBS with 1mg/ml BSA for 2hrs at room temperature. Plates were rinsed with PBS between each step. HEK were plated at 12,500 cells/cm² or 60,000 cells/cm² and cultured in EpiLife supplemented with HKGS and 1.5mM Ca^{++} . Additional cells were plated in untreated wells and cultured in the same medium with 0.03mM Ca^{++} or 1.5mM Ca^{++} . At 48hrs, cells were imaged by phase microscopy and medium changed, followed by protein collection at 72hrs. Protein expression was analyzed using Western blots with the following antibodies: rabbit anti-mouse loricrin (1:1000, Covance, Berkeley, CA), mouse anti-human involucrin (1:100, clone SY5, LabVision, Fremont, CA), mouse anti-human keratin 10 (1:400, clone DE-K10, Dako, Denmark), rabbit anti-human actin (1:500, I-19, Santa Cruz Biotechnology), and mouse anti-human filaggrin (1:1000, clone AKH1, gift of Dr. Beverly Dale-Crunk, University of Washington, Seattle, WA).²⁴ Secondary antibodies were HRP-

conjugate goat anti-mouse IgG and goat anti-rabbit IgG (1:2500, Jackson), and Western Lightning was the chemiluminescent agent (PerkinElmer, Boston, MA).

Rafted organ culture (ROC) model

This model has been previously described by Knowles, et al.¹⁶ Briefly, a protein-modified polyHEMA rod made using a 1.5mm biopsy punch was inserted into a 4mm diameter punch biopsy of human neonatal foreskin obtained from the Infant and Maternal Care Unit at the University of Washington Medical Center as approved by the University's Human Subjects Review Board. The resulting construct was cultured at the air-medium interface on a metal mesh raft, in DMEM/F-12 basal medium supplemented with 0.6% BSA, 1% nonessential amino acids, and antibiotics. A three gas incubator was used with 40% O₂ and 5% CO₂. At 6 days, the samples were fixed in PBS-buffered 10% formalin and paraffin embedded for histological evaluation. The resulting tissue-biomaterial construct was stained using hematoxylin and eosin (H&E) and immunohistochemical techniques. Primary antibodies employed included: rabbit anti-mouse loricrin (1:250, Covance), mouse anti-human keratin 10 (1:50, clone DE-K10, Dako), mouse anti-human keratin 16 (1:500, NeoMarkers), and mouse anti-human proliferating cell nuclear antigen (PCNA, 1:200, Dako). Antigen retrieval was performed as dictated by the manufacturer. Secondary antibodies were biotinylated horse anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA) and biotinylated goat anti-rabbit IgG (1:200, Vector). The ABC peroxidase detection method (Vector) was used with SigmaFast 3,3'-diaminobenzidine (Sigma) as the substrate. Samples were subsequently treated with a methyl green nuclear counterstain.

ROC model data analysis

After staining and slide mounting, cellular responses were analyzed using light microscopy. In total, 18 ROC samples were evaluated (4-8 samples per treatment condition). Inherent within the ROC model is a degree of sample loss and experimental variability. Examples include: failure of the rod to transverse the entire tissue, orientation within the paraffin block leading to suboptimal sectioning, and loss of polymer section during staining. As a benchmark for analysis, only sections with 5 infiltrated polymer pores were included in the analysis. We used H&E to evaluate cellular infiltration into the rod, by counting total number of pores infiltrated and average cells per pore in one section. For immunohistochemical staining analysis, we counted the total number of infiltrated pores per section and the total number of positively-stained pores per section. Epithelial cell responses were quantified as a ratio of positively-stained pores over total number of infiltrated pores.

Statistical analysis

Data were analyzed for significance using a one-way analysis of variance (ANOVA) and a Tukey's post-hoc test for pairwise comparison. The software used was SPSS for Windows and significance set at 95% confidence.

Results

Activity of Jagged-derived peptide

Jagged-1 was originally isolated as a mammalian ligand that activates the Notch-1 receptor. The activation domain of Jagged-1 has now been mapped to the N-terminal extracellular region of the protein, as a specific peptide derived from the DSL-domain of Jagged-1 has been shown to mediate the activation of Notch signaling.¹⁴ Therefore, we initially explored the possibility of harnessing a DSL-containing peptide to activate the Notch pathway from a biomaterial instead of the full-length Jagged. Previous studies have indicated bioactivity of these DSL-containing peptides in influencing cell fate.^{14,19} We designed a peptide based on

this previous work with a hexaglycine and lysine tail for direct immobilization purposes (Jag_DSL). However, when HEK were plated on the polyHEMA-immobilized Jag_DSL, no increase in Notch/CBF-1 signaling over the control peptide (Sc_Jag) was seen at immobilization concentrations of 250 and 1000 $\mu\text{g/ml}$ (figure 1a). To verify the presence of the ligand on the surface, the Jag_DSL-modified polyHEMA was analyzed using ESCA (figure 1b). Since polyHEMA contains no nitrogen and CDI treatment introduces a unique, identifiable nitrogen peak, peptide immobilization can be monitored by nitrogen content analysis. When the polyHEMA was treated with CDI alone (no peptide), the nitrogen 1s peak in ESCA was observed as a doublet due to imidazole nitrogen, and this doublet disappeared with the presence of other nitrogen states introduced by immobilized protein/peptide.²¹ At immobilization concentrations of 500 and 2000 $\mu\text{g/ml}$, there was a broad nitrogen 1s peak, indicating peptide immobilization (figure 1b). In addition, total nitrogen content of the surface also increased (8.4, 8.6, 10.7% for 0, 500, 2000 $\mu\text{g/ml}$ respectively), confirming immobilization. This peptide was further tested in luciferase assays using REEC that had previously shown Notch activation when plated on bound Jagged-1.³ Using REEC transfected with the Notch/CBF-1 reporter plasmid, the peptide showed no Notch/CBF-1 activation when the cells were plated on the peptide adsorbed to TCPS (0-500 $\mu\text{g/ml}$ precoat) or soluble in solution (0-10 μM) (data not shown). In addition, the soluble peptide (1 μM) was unable to block signaling induced by surface-bound Jagged-1 (data not shown). These experiments demonstrate that the Jag_DSL peptide lacks bioactivity with respect to the canonical Notch pathway.

Direct versus indirect immobilization of Jagged-1/Fc to polyHEMA

We have previously employed an indirect immobilization approach for surface modification with Jagged-1. In that approach, an anti-Human Fc antibody was first covalently attached to polyHEMA. The surface was then treated with Jagged-1/Fc, thus indirectly immobilizing the Jagged-1 to polyHEMA through the anti-Human Fc antibody.³ To explore the activity of Jagged-1 immobilized directly to polyHEMA, we activated spun cast polyHEMA surfaces with 20mM CDI and then treated them with Jagged-1/Fc to immobilize the protein to polyHEMA with a carbamate linkage. At high concentrations of Jagged-1 (500nM immobilization concentration), the protein displayed statistically significant Notch signaling activity when HEK transfected with the CBF-1 luciferase reporter were plated on the surface (figure 2a). However, this increase in luciferase activity was small: 1.5 fold over that observed with the control human Fc. ESCA analysis showed that the protein was immobilized to the surface at the concentration ranges used (figure 2b). As expected, the imidazole doublet was no longer seen, and the nitrogen content of the surface increased (8.4, 10.6, 11.9% for 0, 100, 500nM respectively). In contrast, when HEK were plated on a polyHEMA surface first modified covalently with anti-Fc (15 $\mu\text{g/ml}$) and then Jagged-1/Fc (10nM), a 3-fold increase in Notch signaling was observed (figure 2c). Therefore, the indirect immobilization technique resulted in greater signaling at lower Jagged-1 concentrations than direct immobilization, and was used for all subsequent experiments.

Quantification of Adsorbed Protein and Notch Activation

Utilization of the BCA assay allowed us to quantify the surface concentration of Jagged-1/Fc adsorbed to the surface of TCPS via indirect and direct immobilization methods. As seen in figure 3a, the dependence of Jagged-1/Fc surface concentration (ng/cm^2) on precoat concentration (nM) was very similar for both direct and indirect adsorption methods. For both methods, surface concentration quickly approached 150 ng/cm^2 , indicating that monolayer coverage was likely achieved.

Analyzing the influence of precoat concentration on Notch activation, the indirect adsorption of Jagged-1/Fc yielded activation of the signaling pathway at much lower

concentrations compared to the direct adsorption method (figure 3b). By combining the data from the BCA assay and the Luciferase assay, we observe the indirect adsorption method activates Notch signaling more potently at lower surface concentrations than the direct adsorption method (figure 3c). We hypothesize that this potency may be a result of orientation or clustering of the ligand.

Bound Jagged-1 induces expression of intermediate- and late-stage differentiation markers

In previous studies using REEC, surface bound Jagged-1 was shown to increase expression of intermediate- (K10, involucrin) and late-stage (filaggrin) differentiation markers.³ In preparation for the ROC model, we examined the effect of surface bound Jagged-1 on HEK differentiation. Similar to results seen in REEC, HEK cultured in high calcium conditions (1.5 mM Ca^{++}) show increased clustering when plated on the Jagged-1/Fc bound to TCPS compared to Fc alone (figure 4). However, this phenomenon was only seen at lower plating concentrations (12,500 cells/cm²) and not at higher densities (60,000 cells/cm²) (figure 4). The change in cellular morphology correlated to a change in differentiation marker expression. Cells plated on bound Jagged-1 at 12,500 cells/cm² and high calcium conditions show increased expression of involucrin, K10 and loricrin compared to controls (figure 5). However, at a plating density of 60,000 cells/cm², no change in protein expression was seen between treated and control groups. Filaggrin expression was not seen in any culture.

Fabrication and characterization of polyHEMA implants

PolyHEMA rods were fabricated for use in the ROC model (figure 6). Non-porous polyHEMA was polymerized between two glass plates and rods were punched from the resulting slabs (figure 6a and 6b). Porous polyHEMA was prepared using a sphere-template approach.²⁰ In this process, sifted PMMA beads (61-75 μm) were sintered together, followed by infiltration of the matrix by polyHEMA. After solubilizing the PMMA with acetone, the resulting porous polyHEMA construct was punched into rods (5mm long) using a 1.5mm diameter biopsy punch. These scaffolds showed a highly regular and interconnected pore structure (figure 6c and 6d).

Implantation in rafted organ culture model

For evaluation in the ROC model, both porous and non-porous polyHEMA rods were covalently modified using CDI chemistry with glycine or rabbit anti-human Fc. The samples treated with anti-human Fc were subsequently treated with human Fc or Jagged-1/Fc. Following modification, the constructs were implanted into human foreskin tissue and cultured for 6 days. When non-porous polyHEMA was implanted into the foreskin tissue, the epithelial response was identical in all treatment groups. Epithelial cells migrated along the dermis (marsupialization) at the biomaterial-tissue interface (figure 7a and 7b), and showed neither cellular integration nor epithelial interaction with the surface, consistent with previous results.¹⁶ In contrast, the porous polyHEMA rods demonstrated integration with the adjacent epithelium (figure 7c and 7d). The shape of the epithelial clusters and remnants of the polymer observed indicated that the migrating epithelial cells entered and filled the spherical pores.

The spherical pores filled with epithelial cells provided a controlled microenvironment to measure the influence of a Jagged-1 surface modification on epithelial differentiation. We thus compared the foreskin keratinocyte response to porous polyHEMA rods modified with three treatments: CDI + glycine, CDI + anti-Fc + human Fc, and CDI + anti-Fc + Jagged-1/Fc. To evaluate the effect of a Jagged-1/Fc surface modification on epithelial differentiation, marker expression for proliferation (PCNA), intermediate-stage differentiation (K10), activation during wound-healing (K16), and late-stage differentiation (loricrin) within the

pore microenvironment was determined (figures 8 and 9b). There was a striking decrease in the ratio of pores stained for proliferation (PCNA) on the Jagged-1/Fc-modified surfaces compared to the two control surfaces. On the other hand, intermediate-stage differentiation (K10) analysis and activation of wound-healing (K16) analysis revealed a significantly higher ratio of pores stained in Jagged-1/Fc rods compared to glycine- and human Fc-modified rods. However, no statistical significance was seen when evaluating differences in the ratio of pores stained for late-stage differentiation marker, loricrin, in the three different samples. In summary, modification of polyHEMA with Jagged-1 decreased proliferation, increased intermediate differentiation marker expression, and induced a wound healing activation state (figure 9b). When cellular integration into the pores, including total number of infiltrated pores and average nuclei per pore were measured, there were no differences seen between treatment groups (figure 9a). These results indicated that modification of polyHEMA surfaces with Jagged-1/Fc encourages the differentiation of epithelial cells within the porous microenvironment but does not appear to affect rate of epithelial migration.

Discussion

In this study, we have shown that effective Notch signaling from a biomaterial is dependent on ligand identity and presentation, with full-length Jagged-1 indirectly immobilized to the surface showing greatest potency. Bound Jagged-1 was shown to elicit Notch signaling and differentiation in human-derived keratinocytes. This response, including tight clustering and stratification, was similar to that seen previously with rat keratinocytes.³ However, the clustering and differentiation was keenly sensitive to cell density. Finally, in a rafted organ culture model, Jagged-1 modified polyHEMA was shown to decrease cellular proliferation and increase keratinocyte differentiation. These results indicated that modification of polyHEMA surfaces with Jagged-1/Fc encourages the differentiation of epithelial cells within the porous microenvironment but not migration.

Also interesting to note is the increased potency displayed by the affinity-immobilized Jagged-1 compared with direct immobilization. It is hypothesized that this stems from two factors: orientation and clustering of the protein. By first immobilizing an anti-Fc antibody and then adding the Jagged-1/Fc, the Notch ligand is bound by its Fc region, thus uniformly orienting the protein and allowing the receptor binding region to freely interact with the Notch receptor on the surface of the cell. In a direct immobilization, the bond is formed at random free primary amines within the Jagged-1/Fc protein, giving a random orientation and rendering some receptor binding sites inaccessible. As for the clustering hypothesis, previous work has shown that a soluble ligand acts as an agonist when dimerized by an antibody.¹⁰ In our system, the immobilized anti-Fc can potentially bind two Jagged-1/Fc proteins, thus giving an inherent clustering of the ligand, which the direct immobilization scheme lacks. Also of interest is the evaluation of various ligands immobilized to the biomaterial. The DSL-containing peptide used in these experiments did not show bioactivity with regards to the canonical Notch pathway. Whether immobilized or soluble, the peptide did not activate or block the Notch/CBF-1 pathway. Previous studies have indicated bioactivity of DSL-derived peptides.^{14,15,25,26} However, with limited studies, different cell types, and varied readouts measuring Notch activation, it is difficult to draw any conclusions on the effect or mechanism of the peptide. Some have suggested that the high concentrations used in DSL-containing peptide studies results in aggregates, which enhance bioactivity.²⁷ Peptide synthesis inherently gives batch-to-batch variation. The presence of the cysteine in the DSL-containing peptide introduces additional complexity with the possibility of disulfide and hydrogen bonds in the final product. All of these considerations make it difficult to draw any conclusions concerning the DSL-derived peptide without further characterization.

By conducting our tests in a human keratinocyte cell system, the effects of bound Jagged-1 that we observed are more relevant to therapeutic applications than with previously studied rat keratinocytes.³ However, the demonstration of increased intermediate- and late-stage differentiation marker expression in HEK plated on bound Jagged-1 is comparable to the previously published effect on rat esophageal epithelial cells.³ The HEK cells on bound Jagged-1 show increased K10, involucrin, and loricrin expression compared to their control. Unexpectedly, filaggrin expression did not correlate with loricrin expression. This has been seen before by Rangarajan et al., where overexpressed Notch^{IC} in mouse keratinocytes showed substantial increases in K1 (the basic analogue of K10) and involucrin, a slight increase in loricrin, and no change filaggrin expression.²⁸ *In vivo*, keratinocyte-specific Notch^{-/-} mice showed increased filaggrin and loricrin expression, whereas K1 and involucrin was unaffected.²⁸ However, they later show that Caspase 3 ^{-/-} mice have decreased loricrin and filaggrin expression in the mouse epidermis, with no change in K1.²⁹ Caspase 3 production is dependent on Notch activation and also activates the protein kinase C α isoform (PKC- α).^{29,30} Protein kinase C activation has been shown to influence the epidermal cell transition from spinous to granular.³¹ We have presented here and in previous studies³ that biomaterial-bound Jagged-1 activates Notch/CBF-1, induces Caspase 3 gene transcription, and results in upregulation of intermediate- and late-stage differentiation makers. Thus, it is possible that biomaterial-bound Jagged-1 promotes late-stage differentiation through Notch $\rightarrow$ CBF-1 $\rightarrow$ Caspase 3 $\rightarrow$ PKC. Bound Jagged-1 may also increase intermediate-stage differentiation, though a CBF-1-independent Notch pathway as involucrin expression has been shown to increase upon Notch activation in a CBF-1-independent manner.²⁸

Also of note was the dependence of the effect of bound Jagged-1 on cell density. The promotion of differentiation seen at a lower plating density (12,500 cells/cm²) did not translate to the higher plating density (60,000 cells/cm²). Considering that Notch is normally a cell-to-cell signaling event, the impact of cell density seems intuitive since endogenous Notch is still active in the system. Previous work has shown that homotypic interactions by Delta-1 (a Notch ligand) protects the keratinocytes from Notch-induced differentiation.³² In our system, the higher cell density perhaps results in homotypic interactions and serves as a protection to prevent Notch signaling by the material bound Jagged-1/Fc.

As a bridge from *in vitro* analysis to a full *in vivo* study, we have begun to analyze the Jagged-1 modified biomaterial in a rafted organ culture model (ROC). As seen above, when a porous implant is inserted into a foreskin biopsy and cultured, the epidermal cells penetrate the pores of the biomaterial. Infiltration of the pores was not dependent on surface modification, but Jagged-1 modified polyHEMA induced decreased proliferation and increased intermediate differentiation marker expression. This correlates to *in vitro* data and provides insight into the potential *in vivo* response.

In these studies, we have built on our previous work in developing Notch-signaling biomaterials. The effects of varying both immobilization scheme and ligand along with cell density effects on Notch signaling have been described. In addition, the effect of Jagged-1 on human keratinocyte differentiation *in vitro* and the epithelial response in an organ culture model were evaluated. Our findings suggest that Notch-signaling biomaterials provide a robust approach to control keratinocyte differentiation. Because Notch signaling plays a role in controlling numerous cell fate choices, these Notch-signaling biomaterials have the potential to be applied to other progenitor and stem cells technologies, possibly resulting in widespread use in both tissue engineering and regenerative medicine.

Acknowledgments

The authors gratefully acknowledge the assistance of Buddy Ratner and his laboratory, particularly Andrew Marshall and Misty Noble. In addition, Richard Presland, Rima Kulikauskas, Phillip Fleckman, and Lucy Liaw have provided invaluable assistance with reagents and experimental discussion. The surface analysis experiments were done at NESAC/BIO (EB-002027) with the assistance of Lara Gamble. Funding was provided by the Singapore-University of Washington Alliance (SUWA, A*Star of Singapore) and University of Washington Engineered Biomaterials (EEC-9529161).

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Figure 1. Direct immobilization of DSL-containing peptide on polyHEMA ($\pm$ s.d.). a) CBF-1 luciferase assay for HEK plated on polyHEMA-immobilized peptide. b) Detailed Nitrogen 1s region from ESCA analysis of Jag_DSL immobilized to polyHEMA.

Figure 2.

Immobilization of Jagged-1/Fc to polyHEMA (* $p < 0.05$, $\pm$ s.d.). a) CBF-1 luciferase assay of HEK plated on Jagged-1/Fc directly immobilized on polyHEMA. b) Detailed Nitrogen 1s region from ESCA analysis of Jagged-1/Fc directly-immobilized on polyHEMA. c) CBF-1 luciferase assay of HEK plated on Jagged-1/Fc indirectly immobilized to polyHEMA through rabbit anti-human Fc (15 μ g/ml).

Figure 3.

Immobilization of Jagged-1/Fc to TCPS (* $p < 0.05$, $\pm$ s.d.). a) BCA assay determining surface concentration vs. precoat concentration for direct and indirect immobilization of Jagged-1/Fc. b) CBF-1 luciferase assay for HEK plated on Jagged-1/Fc directly and indirectly immobilized on TCPS. c) Combination of plots in a & b demonstrating Notch activation dependence on Jagged-1/Fc surface concentration.

Figure 4.

Phase micrograph of HEK cultured in 0.03mM Ca^{++} (LC) or 1.5 mM Ca^{++} (HC) after 48hrs on TCPS-bound human Fc (bFc) or TCPS-bound Jagged-1/Fc (bJag).

Figure 5.

Western blots of protein lysates from HEK 72hrs after plating on various ligands (LC: 0.03mM Ca^{++} , NT: not treated, bFc: bound human Fc, bJagged: bound Jagged-1/Fc, HUFS: human foreskin extract; NT, bFc, bJagged were cultured with 1.5mM Ca^{++}).

Figure 6.
SEM images of polyHEMA rods; a&b) Non-porous polyHEMA, c&d) Porous polyHEMA.

Figure 7. Histological analysis of a non-porous and porous polyHEMA rod implanted into human foreskin tissue (H&E stain). a) implanted solid polyHEMA rod (rod boundary indicated by solid black line), b) higher magnification of a), c) implanted porous poly(HEMA), d) higher magnification of c).

Figure 8. Immunohistochemical analysis of a porous polyHEMA rod with Jagged-1/Fc surface modification implanted into human foreskin tissue. a) PCNA immunostain (bar 50µm). b) K10 immunostain (bar 50µm). c) K16 immunostain (bar 50µm). d) Loricrin immunostain (bar 50µm).

Figure 9. Quantitative analysis of keratinocyte response to surface modified polyHEMA rods (* $p < 0.05$, $\pm$ s.e.). Values are average $\pm$ standard deviation. a) Cellular infiltration into the implanted rod. b) Differentiation of pore-infiltrating keratinocytes.